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Review

Planar chromatography coupled with spectroscopic techniques

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Abstract

Recent progress in the combination of planar, or thin-layer chromatography (TLC) with a variety of modern spectroscopic techniques is reviewed. The utility of TLC for separation followed by mass spectrometry, with a variety of ionisation techniques, is illustrated with reference to a wide range of compound types including drugs, natural products, industrial chemicals, pesticides and dyes. Applications of the use of TLC in combination with Fourier transform infrared spectrometry (FTIR), near infrared spectroscopy and FTIR photoacoustic spectroscopy are discussed, together with the use of TLC with Raman spectroscopy. The use of conventional fluorescence spectroscopy and fluorescence line narrowing spectroscopy as an aid to compound identification is described. Lastly the coupling of TLC with other chromatographic techniques followed by spectroscopic evaluation is briefly considered.

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1. Introduction

Thin-layer, or planar, chromatography (TLC) remains one of the most widely used of all the chromatographic techniques. However, because of the ease of use of planar techniques many of the applications to which it is put are not necessarily performed by 'chromatographers'. For example TLC still finds wide use in synthetic chemistry as a convenient means of monitoring reactions, etc. Indeed in many areas TLC remains the technique of choice for simple, rapid qualitative separations.

However, whilst these qualitative applications have remained essentially unchanged since the development of this type of chromatography, instrumental TLC has continued to evolve and is now a highly developed quan-

titative analytical tool. Nevertheless, it is often the case that, having separated the components of a mixture, identification of an unknown, or the confirmation of the structure of a component identified solely on the basis of chromatographic properties, is required. Unsurprisingly therefore analytical chemists have sought to maximise the information that can be obtained from TLC separations in the same way that has been seen for other chromatographic techniques (e.g. LC, SFC and GC). Here we will review progress in the combination of planar chromatography with mass spectrometry (MS), infrared (IR), Raman and fluorescence spectroscopy together with those approaches where LC–TLC has been used with one of these techniques (e.g. LC–TLC–FTIR).

1.1. Special features of TLC

Before discussing the ways in which spectroscopic information can be obtained following separation by TLC some discussion of the particular features of planar techniques that either aid or complicate such procedures is required. Obvious advantages of planar chromatographic techniques include speed, ease of use, economy, the ability to analyse many samples in parallel and, as the plates are not used again, less need for sample clean-up. The preeminence of TLC as qualitative screening technique is also noteworthy, together with the fact that a qualitative evaluation can be followed by subsequent quantification, without the need to perform another analysis. However, the major attribute that distinguishes TLC and other planar techniques from column chromatography [with the possible exception of the forced flow planar techniques (e.g. overpressure TLC)] is that separation and detection are discontinuous ('off-line'). This is considered by many to be a limitation, but in fact can also be seen to be a unique advantage for TLC. Thus, many plates can be run simultaneously for subsequent evaluation, and the presence of the analyte in the sample and the quality of the separation can be determined qualitatively prior to scanning densitometry etc. Secondly, such an approach readily enables the analyst to concentrate on a single component of a mixture without the need to wait for other extraneous substances to elute from the column. On the other hand, because the whole chromatogram is contained between the origin and the solvent front the analyst has a much greater chance of observing the presence of unknowns that might be missed by column chromatography due to either non-elution from the column or recovery unretained in the solvent front.

Another feature of TLC, discussed in more detail by Poole and Poole elsewhere in this volume, is the potential to perform multidimensional and multimodal chromatography in order to increase selectivity and obtain the best possible separation. In many ways techniques such as 2-D TLC can be considered to be the equivalent

of column switching in LC, but with the advantage that the process is performed on every component of the mixture, and without the technical difficulty often associated with column switching.

Because chromatography and spectroscopic evaluation are not directly linked, or 'hyphenated', few compromises have to be made with regard to the choice of solvent system employed for the separation. Thus chromatographic selectivity and efficiency, etc., are not compromised by the needs of the detector. The plate itself also serves to store the separation. This is particularly advantageous as it means that the separation may be performed on a site some distance away from the laboratory where the plate is evaluated. Certainly, where the analytes are stable, we have experienced no difficulty in obtaining mass spectra after having mailed plates from one site to another. The fact that the sample is static on the plate, rather than moving with the flow of the mobile phase as in LC, is also helpful, placing less demand on the spectrometer and reducing the technical difficulties involved in ensuring that the component of interest is the one which is actually studied.

2. TLC-MS

The use of TLC with mass spectrometry has been practised for many years with the elution of the analyte from the sorbent and subsequent introduction of the isolated material into the ion source of the spectrometer. Whilst technically undemanding, and undoubtedly effective, this sort of procedure is inelegant, time consuming and tedious to perform. In addition there is the danger of introducing contaminants during the process of eluting the analyte from the silica, and the possibility of losses during sample handling. In general approaches leading to spectra being obtained directly from the sorbent, without the need for elution, are the subject of this section. In addition, as the TLC-MS literature has been well reviewed on a number of occasions [1–3] this section concentrates on developments in, and applications of, the methodology over the

last decade. In addition the review of the literature has been selective and based largely on papers published in English. For a brief overview of early applications involving the recovery of the analyte from the stationary phase prior to MS, and for a comprehensive review of the early TLC–MS literature, the excellent review provided by Busch [1] is recommended. As the TLC–MS literature is now quite extensive the area has been reviewed firstly on the basis of the ionisation technique employed and secondly, if the topic has required it, by compound class. The use of TLC–MS has been reviewed here at some length, with both mass spectroscopy and chromatographic procedures described in detail in order to provide the reader with a clear view of the requirements of the technique.

2.1. Methodological approaches in TLC–MS

A number of different approaches can be employed in order to obtain mass spectra from substances separated by planar chromatography. If the recovery of the analyte from the stationary phase is ignored the first of these, and the most readily implemented, is the removal of the stationary phase from the zone of interest followed by introduction into the ion source of the spectrometer. Stable, volatile, analytes can be determined by electron impact (EI) ionisation after volatilisation from a heatable probe tip. Less volatile, or unstable, analytes can be ionised by secondary ion mass spectrometry (SIMS) or by fast atom bombardment or liquid secondary ion mass spectrometry (FAB/LSIMS) after mixing the stationary phase with a suitable liquid matrix. The advantages of this approach under particular circumstances are manifold. Certainly this technique is very cost effective as there is no need to modify the spectrometer, or the chromatography. Thus, if there are likely to be relatively few applications of TLC–MS in a laboratory this manual type of TLC–MS can still be performed without the need to justify the expense of a purchasing or constructing a specially adapted probe. In addition, if there are only a few components on the plate which are of interest, excising the relevant zones may actually

be the most appropriate and least time consuming method.

The alternative methodology is essentially an instrumental approach and employs the use of probes or plate scanners specifically designed for working with planar separations. The instrumentation that has been developed for TLC–MS has recently been reviewed in detail by Busch [1] and there appear to have been few developments in the intervening period. Thus Ramaley et al. in two papers [4,5] have described a plate scanner for TLC–MS, based on a Finnigan-MAT 4000 quadrupole mass spectrometer, in which the analytes are thermally evaporated into a stream of gas which carries them into the source of the mass spectrometer for chemical ionisation (CI) MS. Volatilisation was achieved using either a pulsed CO₂ laser or high intensity incandescent lamps. Using stepping motors the plate (1 × 10 cm) could be moved at a constant speed, evaporating each spot in turn, to provide a chromatogram. Some loss of chromatographic resolution was noted [4] in the mass chromatograms obtained with this device whether desorption was by heating with the lamps or was obtained using the laser. In the case of desorption with the lamps the problem was considered to be due to the lack of a sharp image of the lamp filament on the plate. It was suggested that this resulted in continued desorption of the analyte from the plate even after it had passed out of the actual image zone. In the case of laser desorption poor peak shape, with pronounced tailing, was attributed to the desorbed material condensing on the cold chamber walls and only slowly desorbing. The addition of three small 10-W heaters improved the results for the laser desorbed material and gave resolution similar to that of the lamp heaters. The authors concluded that resolution “seems limited by redistribution of desorbed material within the plate chamber and by adsorption on cooler surfaces swept by the transport gas before it reaches the ion source” [4]. In the second publication from this group [5] a wide range of compound types were investigated using laser desorption and a range of TLC sorbents. The conclusion of the study was that silanised silica gel gave the best results

in respect to desorption characteristics, followed by polyamide, with the best chromatographic results seen using silica-based surfaces. HPTLC plates were superior to TLC plates in these applications. Interesting and sophisticated though this device was it does not appear to have been commercialised. However, a number of simple direct TLC–MS probes have been produced for use with standard mass spectrometers for TLC–FAB/LSIMS by several manufacturers (e.g. Jeol, VG). These are based on attaching a small portion of the plate (ca. 10×65 mm), essentially one track from a developed plate, to the probe. This segment of plate, after application of an appropriate FAB/SIMS matrix, is then moved through the FAB beam using a stepping motor. This allows the analyst to obtain both mass spectra and a mass chromatogram. A diagram illustrating one such device, is shown in Fig. 1.

By far the most sophisticated developments in TLC–MS scanners have resulted from the work of Busch and his collaborators which have resulted in devices capable of two-dimensional imaging of TLC plates using LSIMS [1–3,6–9]. The optimal use of such devices has required considerable ingenuity to overcome certain of the technical problems associated with LSIMS. Thus, for example, the liquid matrices normally used (such as glycerol, *m*-nitrobenzyl alcohol) provide only a limited amount of time for imaging before spot diffusion occurs degrading the separation. The use of a matrix which is solid until the energy from the ion beam of the mass spectrometer melts it (e.g. sorbitol, threitol) has however, been shown to be excellent at preserving spatial resolution (e.g. see Fig. 2) [10,11]. Such ‘phase transition’ matrices also enable a persistent ion current to be obtained for long periods. These properties have resulted in the

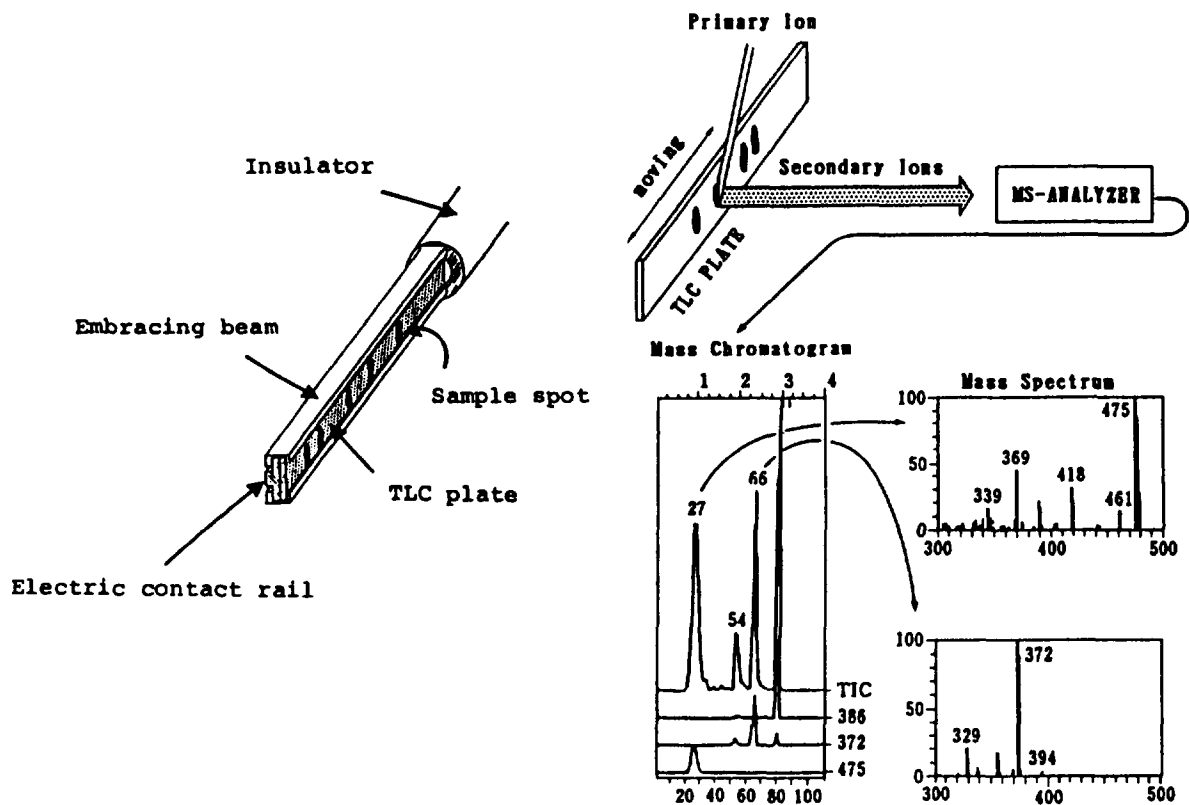


Fig. 1. A schematic representation of a simple probe for scanning TLC–MS taken from Ref. [23].

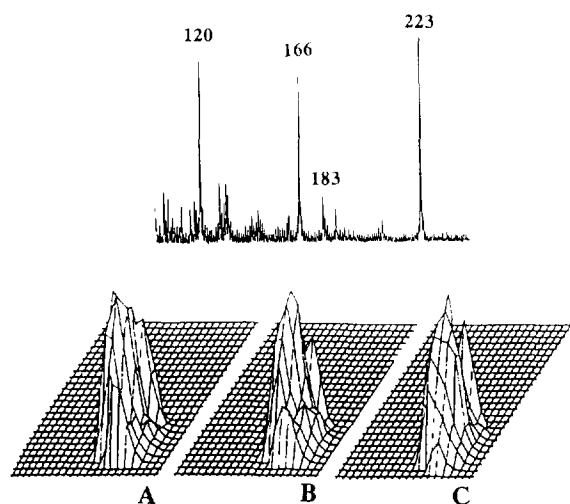


Fig. 2. (Top) positive ion secondary ion mass spectrum of glycylphenylalanine extracted from a TLC plate with sorbitol maintained at its melting point. (Bottom) spatially resolved mass spectral images for the fragment ion at (A) m/z 120, (B) m/z 166 and (C) m/z 223. Adapted from Ref. [11].

production of high quality images (many of the applications described using this technology are discussed below). The use of this purpose built TLC–SIMS scanner in conjunction with an interchangeable optical charge-coupled device (CCD) camera in order to visualise spots in-situ has also been described [7]. It is to be hoped that this device, or one developed from it, will become commercially available at some stage.

One area where more developments in the linking of TLC and mass spectrometry might have been expected is in the area of overpressure layer chromatography (OPLC). After all, in this instance all that is required is to take the eluent from the OPLC directly into the MS via a conventional liquid chromatography interface. However, to date there has only been one brief claim that this coupling has been successfully achieved (with no details) [12].

3. TLC–electron impact mass spectrometry (EIMS)

In general the use of TLC–EIMS for polar involatile compounds, separated on silica gel has

not been particularly successful (see e.g. Ref. [13]). This is due to the strong adsorption of such analytes to the silica, with decomposition occurring as heat is applied to volatilise the analytes. This is clearly less of a problem for relatively volatile analytes, but hardly recommends itself as a robust problem solving technique. Some of these problems can be overcome to some extent by using less adsorbent phases.

Thus, in a series of publications Kraft and co-workers [14–16] have explored the use of EI mass spectrometry (70 eV) for the analysis of a wide range of compound types following separation on polyamide TLC plates. Polyamide is particularly useful for TLC–EIMS work because the analytes are not as strongly adsorbed to the phase compared to silica gel or the other commonly used TLC adsorbents. Analytes separated on polyamide can thus be analysed by EIMS without the need to heat the probe to high temperatures, which would cause the decomposition of the analyte. In this technique the zone of polyamide required for analysis was removed from the backing with a spatula and introduced directly into the quartz sample tip of the direct insertion probe. Washing the polyamide by pre-developing the plates twice in chloroform resulted in low backgrounds. Thus even ion source temperatures of 310°C had no effect on the spectra of the analytes in terms of background from the polyamide [14]. Spectrometry of sub-microgram amounts of a diverse range of analytes was shown in the first report from this group, including nitrophenols, nucleosides, biogenic amines, sterols and dansyl or PTH derivatives [14]. In subsequent studies the technique was extended to amino acids as dansylated methyl esters [15] and mixtures of dansylated dipeptides [16]. In the case of the amino acids derivatisation of the dansyl derivatives with diazomethane to produce suitably volatile derivatives enabled detection of pmole quantities [15]. The TLC–EIMS of the dipeptides [16] similarly involved the use of dansylation and subsequent methylation using diazomethane. 2-D TLC of these mixtures was employed to obtain improved resolution, but even where compounds co-migrated the individual compo-

A further application of TLC–EIMS employing separations on polyamide to identify degradation products of the pesticides bromophos [O,O - dimethyl - O - (4 - bromo - 2,5 - dichlorophenyl)-phosphorothionate] and iodofenphos [O,O - dimethyl - O(2,5 - dichloro - 4 - iodophenyl)phosphothionate] has been described [17]. The pesticides were added to rainwater and either irradiated with artificial light or kept in darkness. After periods of time of up to 96 days samples were taken, subjected to solvent extraction and analysed by TLC. Spots of interest were identified on the plate using an enzymic method for the detection of cholinesterase-inhibiting compounds. Areas of polyamide containing the parent compounds and degradation products were removed from the backing of the plates and inserted into the sample tip of the direct inlet system for mass spectrometry. Two degradation products of bromophos were detected, broxoxon and 4-bromo-2,5-dichlorophenol whilst in the case of indofenphos the compounds iodofenoxon and 2,5-dichloro-4-phenol were identified. The background from the polyamide was low and the detection of nanogram quantities of material was claimed.

Busch et al. [18] have recently described the evaluation of EIMS for the detection of codeine from both Empore silica gel sheets and a conventional HPTLC silica. The appropriate portion of the sheet or silica gel was placed in the sample cup of the direct insertion probe and 10 μ l of methanol added as an extraction solvent. EIMS was performed at 70 eV and a source temperature of 200°C. With 10 μ g of material on the plate the molecular ion (m/z 299) was present in the spectra from both phases together with a fragment ion corresponding to the loss of C_4H_6O . Morphine was also detected by EIMS after in-situ silylation. Ions were detected for the diTMS and monoTMS derivatives (m/z 429 and 357, respectively) together with a fragment ion at 236 a.m.u. (resulting from the loss of both TMS groups and cleavage of the n -C bond). Derivatisation was performed on Empore silica gel, C_8 and C_{18} sheets by taking the excised portion of the plate, putting it in a 1-ml vial and adding 200 μ l of BSTFA–1% TMCS [bis(trimethylsilyl)trifluoroacetamide – trimethyl-

chlorosilane] and heating at 70°C for 20 min. After derivatisation the sample was placed in the probe cup and heated at 400°C in order to obtain spectra. These authors also demonstrated that overlapped components on C_8 Empore sheets could be identified by the summed mass spectra of the compounds evaporated in the EI source using overlapped spots of papaverine and noscapine as examples. The mixed spot was transferred into the sample cup, 10 μ l of methanol added as an extraction solvent and EI spectra obtained in the usual way. In the resulting spectra $[M + H]^+$ ions both for papaverine (m/z 339) and noscapine (m/z 220) were detectable.

One of the more recent applications of the use of EIMS, for the analysis of a mixture of polymer additives, separated on silica gel [19], employed tandem mass spectrometry (MS–MS). This application is described in more detail in the section dealing with TLC–MS–MS.

4. TLC–chemical ionisation mass spectrometry (CIMS)

The use of chemical ionisation (CI) techniques in combination with TLC has not been widely exploited. The most extensive work in this area has been reported by Ramaley et al. [4,5] in conjunction with work on the development of the TLC–MS scanner described above. In the first description of the technique the polycyclic aromatic hydrocarbon (PAH) phenanthrene was used as a model compound to demonstrate the utility of the system [4] with methane as the reagent gas. A pseudo-chromatogram, obtained by scanning at m/z 179 (the $[M + H]^+$), was provided for five 0.5- μ g spots of phenanthrene. A detection limit of 10 ng was suggested. The use of the system with a PAH mixture (1 μ g amounts) was also demonstrated. Subsequent work [5] evaluated the scanner for a wide range of compound types present on silica gel at 10 μ g/spot. Some compounds, such as diphenylamine, biphenyl and benzoquinone were readily observed, whilst others, including catechol, anthranilic acid and cholesterol were undetected. In general the authors concluded that the more

polar the compound the more difficult it was to detect, with acids and alcohols giving low sensitivity and amino acids undetectable. As molecular mass increased, and volatility decreased, detection also became more problematic and compounds with molecular masses above 300 were not observed consistently.

An application of TLC–CIMS to the analysis of crude synthetic reaction mixtures has also been described for monitoring the synthesis of a β -lactam antibiotic [20]. In this work the relevant areas of the TLC plate were touched with a bead of warm (60–70°C) Carbowax 20M, attached to the probe tip of the mass spectrometer. The sticky nature of the warm Carbowax resulted in the removal of silica from the plate enabling mass spectra to be obtained in the usual way. No background was observed from either the Carbowax or the phase used for chromatography when the source temperature was maintained below 220°C. Plastic-backed TLC plates were used and a wide range of stationary phases were found to give good results (e.g. silica gel, alumina, and C₂, C₈ and C₁₈ bonded materials). Improved results were observed when ammonium chloride was added to the samples. Both positive and negative ion CI were performed with ammonia or argon as reagent gases. It was also demonstrated that in-situ ozonisation could be performed on the TLC plates and monitored by CIMS.

5. TLC–liquid secondary ion mass spectrometry (LSIMS), TLC–secondary ion mass spectrometry (SIMS) and TLC–fast atom bombardment mass spectrometry (FAB-MS)

The use of TLC in combination with ‘soft’ ionisation techniques such as SIMS, LSIMS or FAB-MS has proved to be an exceptionally versatile approach that has been applied across a whole range of analytes, including drugs and their metabolites, pesticides, natural products, synthetic chemicals and dyes. To date the bulk of the published studies have involved the removal of the stationary phase from the plate for subsequent mass spectroscopic analysis. However,

with the more widespread availability of simple motorised probes of the type discussed above this situation will undoubtedly change.

5.1. TLC–MS of drugs and drug metabolites

5.1.1. Antibiotics

The use of TLC–FAB-MS for the analysis of a number of antibiotics was one of the first applications of the technique and two such studies were described by Chang et al. [21,22]. For these experiments the probe tip of the mass spectrometer was covered with a strip of double sided masking tape enabling the required area of silica on the plate to be transferred merely by pressing the tip of the probe against it. A few microlitres of a suitable FAB matrix, such as glycerol or thioglycerol, together with a co-solvent (dichloromethane or methanol) were then applied to the silica removed from the plate and the probe placed in the spectrometer. In the first application described by these authors [21] TLC–FAB-MS was used to obtain spectra of three commercially available poultry coccidiostats, lasocoid, septamycin and monensin. A Kratos MS-50 was used, equipped with an M-Scan FAB source and gun. FAB spectra were acquired using fast xenon atoms with thioglycerol as the matrix. Chromatography was performed on silica gel, using ethyl acetate–dichloromethane (70:30, v/v) as eluent. Lasocoid and monensin gave $[M + Na]^+$ ions at m/z 613 and 693, respectively. Septamycin also gave an $[M + Na]^+$ (m/z 937) but in addition gave an intense fragment ion at m/z 875. Less than 0.1 μ g of monensin could be detected by the technique. No problems of increased background etc were noted to result from either the plates or the adhesive tape.

In the second application from this laboratory [22], the analysis of a mixture of bleomycins present in the drug Blenoxane was investigated. These compounds are a family of glycopeptide antibiotics with antineoplastic properties, produced by *Streptomyces verticillus*. These compounds are polar, thermally unstable and have molecular masses of 1400 or more. The compounds present in Blenoxane (bleomycins A₂ and B₂) were chromatographed on silica with

methanol-10% ammonium acetate (1:1, v/v) and two major spots were detected by UV quenching. Positive FAB-MS of the higher R_F components detected in the mixture gave an $[M + H]^+$ of m/z 1425 for bleomycin B_2 . In the case of the spot with the lower R_F two major ions (m/z 1400 and 1414) were obtained corresponding to demethylbleomycin A_2 (produced by degradation of bleomycin A_2 under the FAB conditions) and bleomycin A_2 itself respectively. The authors concluded that TLC-FAB-MS gave comparable results to those obtained by off-line LC-MS analysis. However, they concluded that

TLC-FAB-MS was simpler, more economical and less laborious.

Subsequently Nakagawa and Iwatani [23] showed TLC-LSIMS of three cephalosporins (including cephalixin methyl ester) (Fig. 3) together with a wide range of other compounds (see later). Positive ion mass spectra were obtained in-situ from strips of aluminium-backed silica gel TLC plates attached to a motorised probe constructed by the authors. Glycerol was used as matrix and spectra were obtained using a Hitachi M-68 mass spectrometer. These spectra were superimposable on those obtained for the

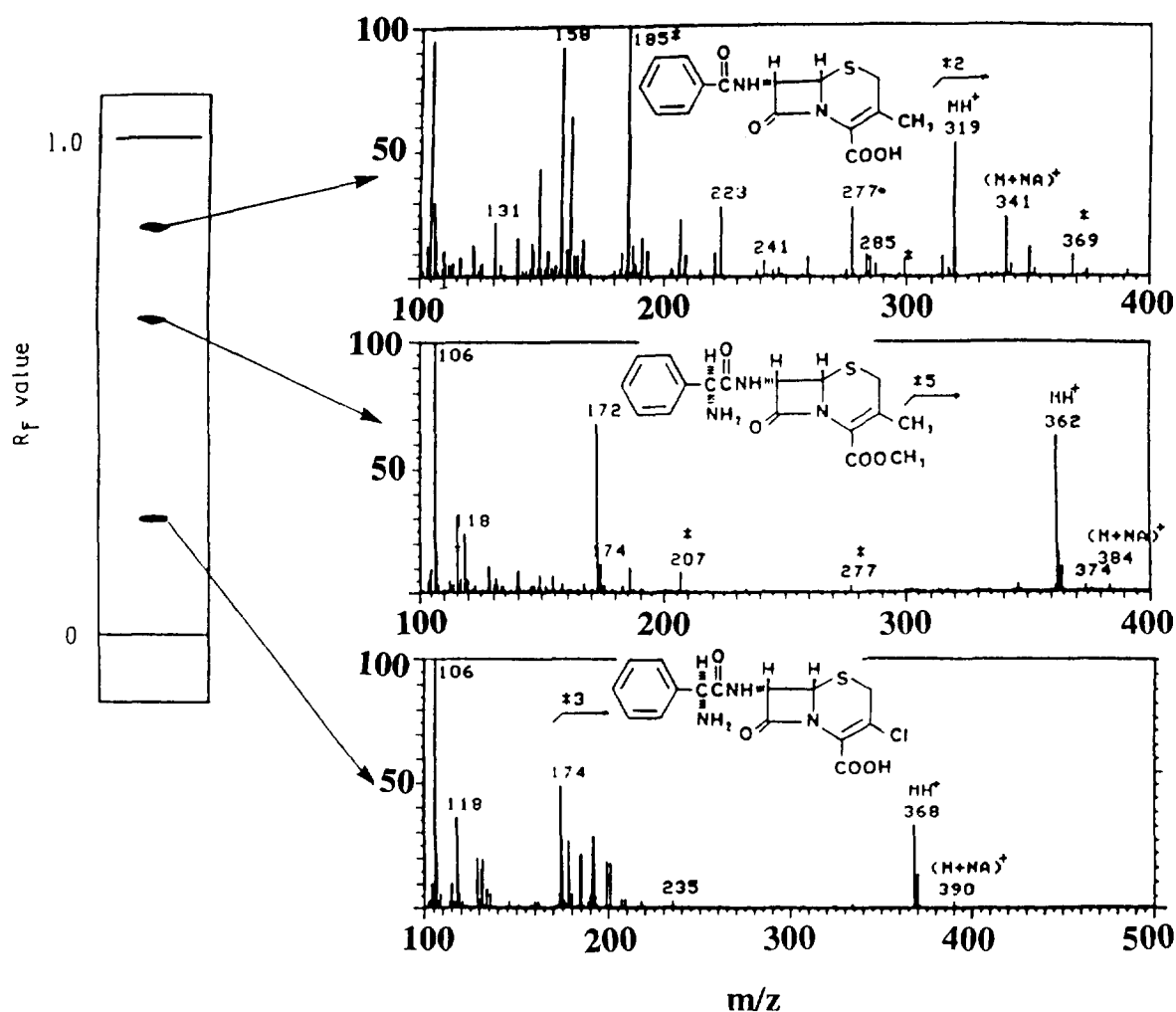


Fig. 3. Scanning TLC-LSIMS of cephalosporins from silica. Adapted from Ref. [23].

standards directly from a conventional silver plate. The instrument was also applied to the scanning TLC–MS of a mixture of glycosides which included tobramycin, apramycin and fradomycin B from aluminium-backed and glass-backed TLC plates and from a homemade sintered glass plate (Fig. 4).

In a series of publications Oka et al. [24–26] have described the use of TLC–FAB–MS for the analysis of antibiotics and dyes. The first example was mainly devoted to methods for in-situ concentration of the analytes [24]. This concentration of the analytes was accomplished by selectively removing the silica around the spot of interest, in a trapezoid shape, and then applying a highly elutropic eluent (methanol) at the broad base of the trapezoid causing the analyte to migrate to the narrow end and there be condensed into a narrow band. This concentrated zone of analyte was then removed for spectrometry. TLC was performed both on silica

gel and C_{18} bonded silica gel plates with subsequent positive ion FAB–MS (xenon, 5 kV) performed using a variety of matrices depending upon the analyte. For example in the case of mycinamycin, IV *m*-nitrobenzyl alcohol gave strong $[M + H]^+$ ions at m/z 696 with as little as $0.3 \mu\text{g}/\text{spot}$ from silica gel and $0.1 \mu\text{g}$ from the C_{18} bonded phase. Similar results were obtained for microcystin LR ($[M + H]^+$ at m/z 995) with glycerol–1 *M* HCl (or 1% aqueous trifluoroacetic acid) (1:1, v/v) as matrix. In this case detection limits were $0.1 \mu\text{g}$ and $5.0 \mu\text{g}/\text{spot}$ from silica gel and C_{18} bonded silica, respectively. For TLC–FAB–MS of viridopentaose B glycerol provided a suitable matrix enabling $[M + Na]^+$ ions at m/z 862 to be obtained for $0.1 \mu\text{g}/\text{spot}$ from silica gel and $1.0 \mu\text{g}/\text{spot}$ for the C_{18} bonded material. The condensation technique was shown to be key in obtaining such low detection limits, increasing sensitivity by 3 to 100 times depending upon the analyte.

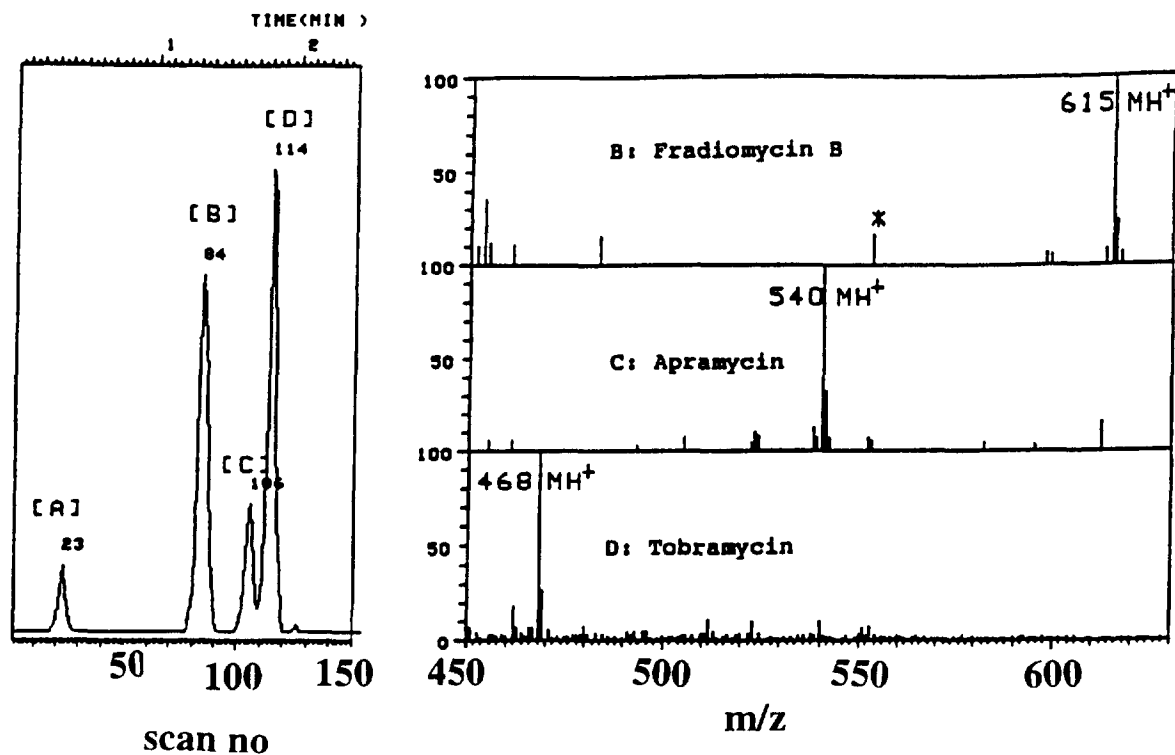


Fig. 4. Scanning TLC–MS of aminoglycosides from silica. Adapted from Ref. [23].

Recent studies on the TLC–MS of antibiotics from this group have provided further demonstrations of the potential of the technique with the example of the analysis of tetracyclines in bovine liver, kidney and muscle [25] and milk [26]. In the tissue study [25], following extraction into McIlvaines buffer and solid phase extraction, the tetracyclines (tetracycline, oxytetracycline, chlortetracycline and doxycycline) were separated on C_8 bonded TLC plates. The eluent used was methanol–acetonitrile–0.5 M aqueous oxalic acid (pH 2.0) (1:1:4, v/v). The same analytes and chromatographic conditions were employed in the subsequent study for tetracyclines in milk [26]. Following chromatographic development the spots of analyte were located by reference to standards run on the same plate (visualised colourimetrically). The areas corresponding to the R_f of the target tetracyclines in the extracts were then subjected to a concentration technique. In this technique the area of silica gel containing the required spot was cut into a trapezoidal shape and a small amount of methanol used to chromatograph the components of interest into the upper base of the trapezoid. The appropriate segment of the plate was then placed upon the TLC holder, FAB matrix (thioglycerol) applied and mass spectra acquired. For mass spectrometry a Jeol JMS-AX505W mass spectrometer was used with xenon at 5 keV and an acceleration voltage of 3 keV. Using the condensation technique to concentrate the analytes prior to spectrometry allowed a reduction in the limit of detection from 5 $\mu\text{g}/\text{spot}$ to 0.1 $\mu\text{g}/\text{spot}$. In the spiked tissues (0.1 $\mu\text{g}/\text{g}$) the $[\text{M} + \text{H}]^+$ ions for tetracycline (m/z 445), oxytetracycline (m/z 461) and chlortetracycline (m/z 479) were detected, whilst doxycycline gave both an $[\text{M} + \text{H}]^+$ (m/z 445) and $[\text{M} + \text{H} - \text{NH}_3]^+$ (m/z 428) ions. In actual samples, where the concentrations of the antibiotics ranged from 0.58 to 11.04 $\mu\text{g}/\text{g}$ more extensive fragmentation was observed including $[\text{M} + \text{H} - \text{NH}_3 - \text{H}_2\text{O}]^+$ ions. Similar results, with identification at the 50 ng/g level, were obtained for the analysis of tetracyclines in milk where TLC–FAB–MS was used as a confirmatory technique for LC.

5.1.2. Alkaloids

The alkaloids choline and muscarine have been identified in mushroom extracts (*Inocybe napipes*) using a range of techniques including TLC–SIMS following separation on cellulose [27]. Chromatography was performed with 1-butanol–methanol–water (58:25:17, v/v/v) and the spots were either analysed directly or the analytes were recovered from the phase and then applied to a silver foil support for positive ion SIMS. Choline was detected as an ion at m/z 104, whilst muscarine appeared at m/z 174. The authors reported that extraction of the spot and SIMS from the silver foil gave higher quality spectra than direct TLC–SIMS by eliminating the high background due to the cellulose. Detection limits of ca. 10 μg for each of the alkaloids were reported.

More recently, a series of reports has described the use of direct FAB–MS, capillary zone electrophoresis, one- and two-dimensional TLC and TLC–FAB–MS to analyse extracts of the rhizome of the medicinal plant *Sanguina canadensis* (Bloodroot) [28,29]. These extracts contained a mixture of benzophenanthridine alkaloids including sanguinarine. TLC both on conventional silica gel TLC plates and on Empore sheets was attempted but only the conventional plates gave satisfactory results. FAB–MS was performed both on material extracted from silica scraped from the plate or directly from spots excised from the plate and applied to the probe tip. The latter approach successfully identified sanguinarine on the basis of the presence of the characteristic ion at m/z 332. Whilst TLC–FAB–MS was possible for the major components separated by LC, signal intensity was lower than that obtained directly from the probe by at least a factor of two. In the case of spots containing smaller amounts of material extracting the signal from the noise became difficult.

The quantitative TLC–LSIMS of the semi-synthetic alkaloid nicergoline (10 α -methoxy-1,6-dimethylergoline-8 β -methanol 5-bromonicotinic acid) with 5-chloronicergoline as an internal standard has been described [30]. Chromatography was performed on silica gel with dichloromethane–acetone–water (100:10:1) as eluent.

Under these chromatographic conditions the analyte and internal standard co-migrated. Spectroscopy was performed by cutting the section of the plate containing the nicergoline and internal standard and attaching it to the SIMS holder of an Hitachi M-80A mass spectrometer. Four different compounds were tried as matrix, with glycerol proving to be the matrix of choice. Using positive ion LSIMS the protonated molecular ions of nicergoline were readily detected at m/z 484 and 486, together with intense fragment ions at m/z 452 and 454 ($[M - OCH_3]^+$). Calibration curves, obtained by plotting the ratio of these nicergoline fragment ions and those of the internal standard were linear over the range 50 to 1000 ng. Good precision and a detection limit of 10 ng were claimed.

Busch et al. [18] used a VG-70SEQ, equipped with a cesium ion gun for LSIMS, and a VG-70SE, fitted with a xenon atom gun for FAB-MS, to obtain spectra for the alkaloids morphine, codeine, noscapine and papaverine after TLC. Spectra were obtained from Empore C_{18}

and C_{18} bonded and silica gel sheets and from conventional silica gel HPTLC plates. From the Empore TLC sheets the best overall results were obtained using a 1:1 mixture of methanol and glycerol. A mixture of *m*-nitrobenzyl alcohol–methanol (67:33) was also generally satisfactory. For both papaverine and noscapine the best results were obtained from conventional silica gel plates with the $[M + H]^+$ ions readily detected. In contrast these ions were barely detectable above the background from the Empore material (12–13 $\mu\text{g}/\text{plate}$).

5.1.3. Benzodiazepines

In their paper on the use of TLC–LSIMS Nagagawa and Iwatanini [23] included an example of the detection and mass spectrometry of rilmazafone and three metabolites (Fig. 5). Spectra were obtained from aluminium-backed silica gel following chromatography using benzene–*n*-propanol–28% ammonia (90:30:1, v/v/v). Glycerol was used as matrix.

An application of TLC–FAB-MS to the identi-

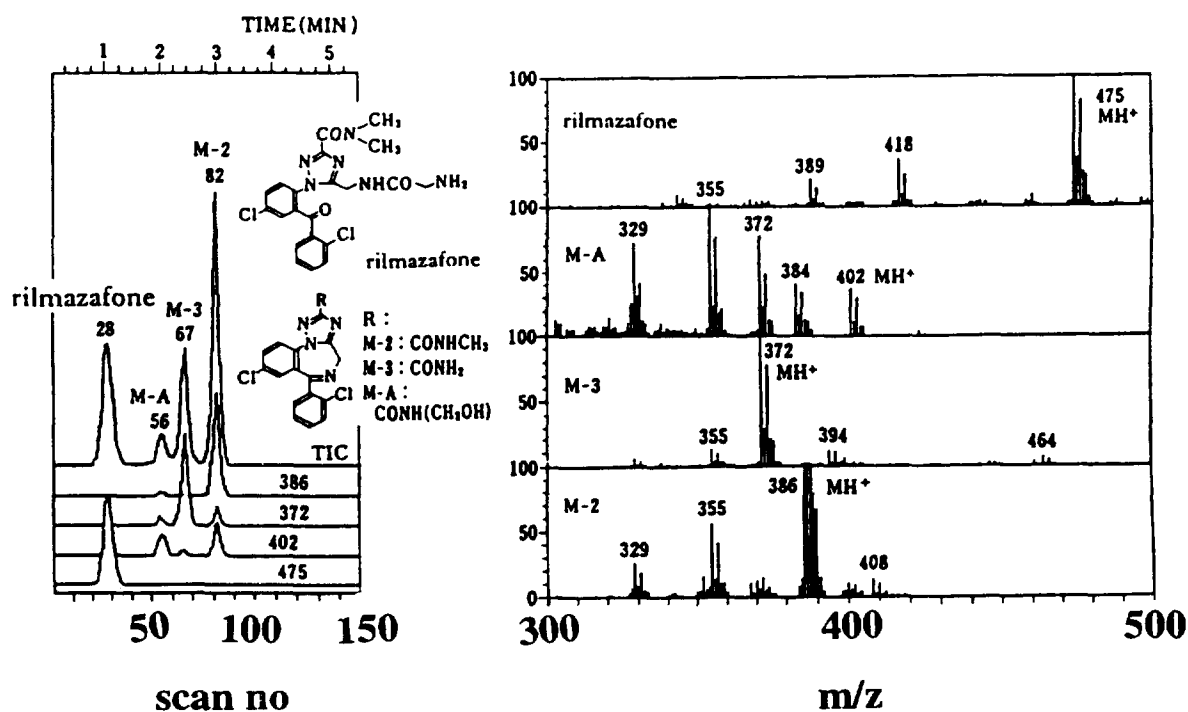


Fig. 5. Scanning TLC–LSIMS of rilmazafone and metabolites from silica gel. Adapted from Ref. [23].

fication of midazolam in plasma extracts following TLC on 3-glycidoxypropyl-treated silica gel TLC plates has been reported [31]. Spectrometry was performed on a Jeol AX505W instrument with xenon at 5 keV. After chromatography, with chloroform–methanol (100:6, v/v) as eluent, the required spots were concentrated on the plate by removing the silica on three sides of the spot in a trapezoidal shape and then using methanol to elute the analyte into a narrow band at the tip of the remaining silica. TLC–FAB–MS of this concentrated zone gave clear spectra showing the $[M + H]^+$ ion at m/z 326. This methodology was applied to a sample obtained from a patient suffering from midazolam intoxication containing 823 ng/ml of the drug (by HPTLC-scanning densitometry at 240 nm). An improvement in the detection limit for midazolam by a factor of 30 was claimed for the TLC–FAB–MS procedure. *m*-Nitrobenzyl alcohol was found to be the optimal matrix for this compound.

5.1.4. Diuretics

Busch and co-workers have described a number of studies using TLC–MS for diuretics [28,32,33]. In the most complete description of this work [32] FAB–MS, in combination with TLC using ethyl acetate–water (100:1.5, v/v) and silica gel, was used to obtain spectra for a total of six diuretics. The authors employed the instrumental TLC–MS approach described earlier which enabled spatially resolved images of the components separated on the plate to be obtained. Threitol was used as the FAB matrix with argon (8 keV) used to obtain the FAB spectra [32]. The compounds investigated were hydroflumethiazide, furosemide, trichlorothiazide, amiloride, hydrochlorothiazide and chlorthalidone. Some experiments were also performed on urine samples spiked with amiloride and hydrochlorothiazole. Ethyl acetate–water (100:1.5, v/v) was employed for the separation of the mixtures, whilst for the urine sample ethyl acetate–methanol–ammonia (85:10:5, v/v/v) was used in order to separate the analytes from endogenous urinary contaminants. In the case of the spiked urines the samples were spotted directly on to the plate

without prior sample preparation. For the majority of the analytes negative ion FAB–MS gave the best results. However, in the case of amiloride positive ion FAB also gave a good response. Quantitative TLC–MS using positive FAB–MS of amiloride, based on the detection of the $(M + H)^+$ ion at m/z 230 was shown. Detection limits of ca. 1–10 ng, a linear range from 10 ng to 1 μ g and a relative standard error of 3–5% were achieved. The same group also reported the use of a time of flight (TOF) SIMS instrument for amiloride hydrochloride (and a sulphonium salt) [34]. The instrument was operated in both ion microscope and ion microprobe modes, with spectra obtained from silica gel and C_8 bonded Empore TLC sheets. Good images of spots of the analytes on the TLC plates were demonstrated. Different detection limits for the two types of phase were reported with the C_8 material giving poorer sensitivity.

5.1.5. Neostigmine and pyridostigmine

Neostigmine and pyridostigmine are quaternary ammonium salts, used clinically for the treatment of myasthenia gravis. Spatial imaging of spots of these compounds following TLC on aluminium-backed silica gel (1-butanol–methanol–water; 4:1:1, v/v) by LSIMS using a custom built instrument has been described [35]. In this study melted sorbitol was layered over the area of interest on the plate to act as a phase transition matrix. The sorbitol was allowed to solidify before the plate was placed inside the mass spectrometer. Once inside the instrument the plate was heated to 59°C which, whilst not high enough to melt the sorbitol (m.p. 93–95°C) was sufficient to maintain a persistent secondary ion signal without excessive spot diffusion. Neostigmine gave an ion at m/z 223, assigned to the intact cation and a further ion at m/z 209 resulting from the loss of a methyl group. Pyridostigmine was detected as the intact cation by the presence of an ion at m/z 181. Imaging of the spots was performed by setting the quadrupole of the mass analyser to pass either the ion at m/z 233 or 181 and moving the manipulator in the XY plane in 0.5 mm increments and good images of the spots were achieved in this way.

The signal in these TLC/imaging experiments was reported to be stable for several hours due to the low volatility of the matrix, but reportedly could take up to 45 min to attain maximal, steady state, values. As a result of this time dependent behaviour the authors recommended the use of internal standards for quantitative work. A quantitative experiment, in which $^2\text{H}_3$ -pyridostigmine was used as an internal standard for pyridostigmine with glycerol as the matrix gave detection limits of ca. 10 ng on the plate.

5.1.6. Phenothiazine drugs

The phenothiazines are a group of compounds with a wide range of pharmacological activities including antiemetic, antipsychoic, antipuritic, antidyskinetic, analgesic and antihistaminic properties. A number of these compounds, including acepromazine, ethopromazine, chlorpromazine, have been the subject of TLC–LSIMS studies [36,37]. The compounds were chromatographed on silica gel with methanol–ammonia (100:1.5, v/v) as solvent. Glycerol or threitol were used as the matrix depending upon the LSIMS experiment with positive secondary ion spectra obtained using caesium ions (8 keV) on a custom built spectrometer. Following chromatography the compounds were detected by UV, the spots excised from the plate, attached to the direct-insertion probe and glycerol added. Representative spectra for some of these compounds are given in Fig. 6. For all but one of the ten compounds investigated the $[\text{M} + \text{H}]^+$ ion formed the base peak in the spectrum. The authors also performed experiments to determine the spatial resolution of the drugs on the silica gel, using either glycerol or threitol as the matrix. To obtain these results the plate was moved using the instruments X and Y manipulators, and changes in the abundance of the selected ion used for detection were monitored. This technique allows the distribution of compounds within an unresolved TLC spot to be determined. Detection limits of 50 to 250 ng were claimed.

5.1.7. Drug metabolites

TLC–LSIMS studies with either manual [38] or instrumental [23] techniques have demonstra-

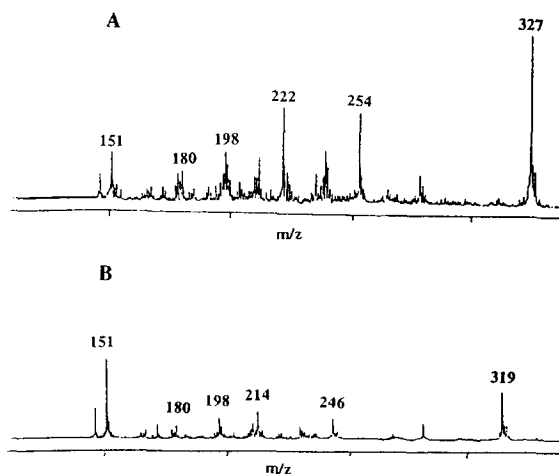


Fig. 6. TLC–SIMS of (A) acepromazine, and (B) chlorpromazine. Adapted from Ref. [36].

ted the effectiveness of the approach for the determination of a range of drug metabolites. In the manual method the phase II conjugated metabolites of *p*-nitrophenol and 4-hydroxyantipyrine were determined [38]. Chromatography was performed on silica gel with 1-butanol–acetic acid–ethanol–water (3:1:1:1, v/v). The analytes were detected by UV and the areas of interest cut out and attached to the probe with double sided masking tape. Glycerol or thioglycerol were used as the matrix. Positive and negative ion spectra were acquired using xenon (8 keV) enabling the identification of the sulphate and glucuronide metabolites of 4-hydroxyantipyrine (a metabolite of the drug antipyrine) produced by *in vitro* incubation with rat hepatocytes. The sulphate was identified as a spot with an R_F of 0.6 by positive and negative ion mass spectrometry with ions at m/z 329 and 307 corresponding to the $[\text{M} + \text{Na}]^+$ and $[\text{M} + \text{H}]^+$ ions and those at m/z 305, 283 and 97 to the $[\text{M} - \text{H}]^-$, $[\text{M} - \text{Na}]^-$ and $[\text{SO}_4\text{H}]^-$ ions respectively (Fig. 7a). The glucuronide was identified as a spot with an R_F of 0.4, also on the basis of positive and negative ion spectra, giving ions at m/z 425 and 379 for the $[\text{M} + \text{Na}]^+$ and $[\text{M} - \text{Na}]^-$, respectively (Fig. 7b). Thioglycerol was used as the FAB matrix in order to obtain spectra of the sulphate metabolite whilst glycerol was employed for the glucuronide. In the same study the TLC–MS of the sulphate and glucuro-

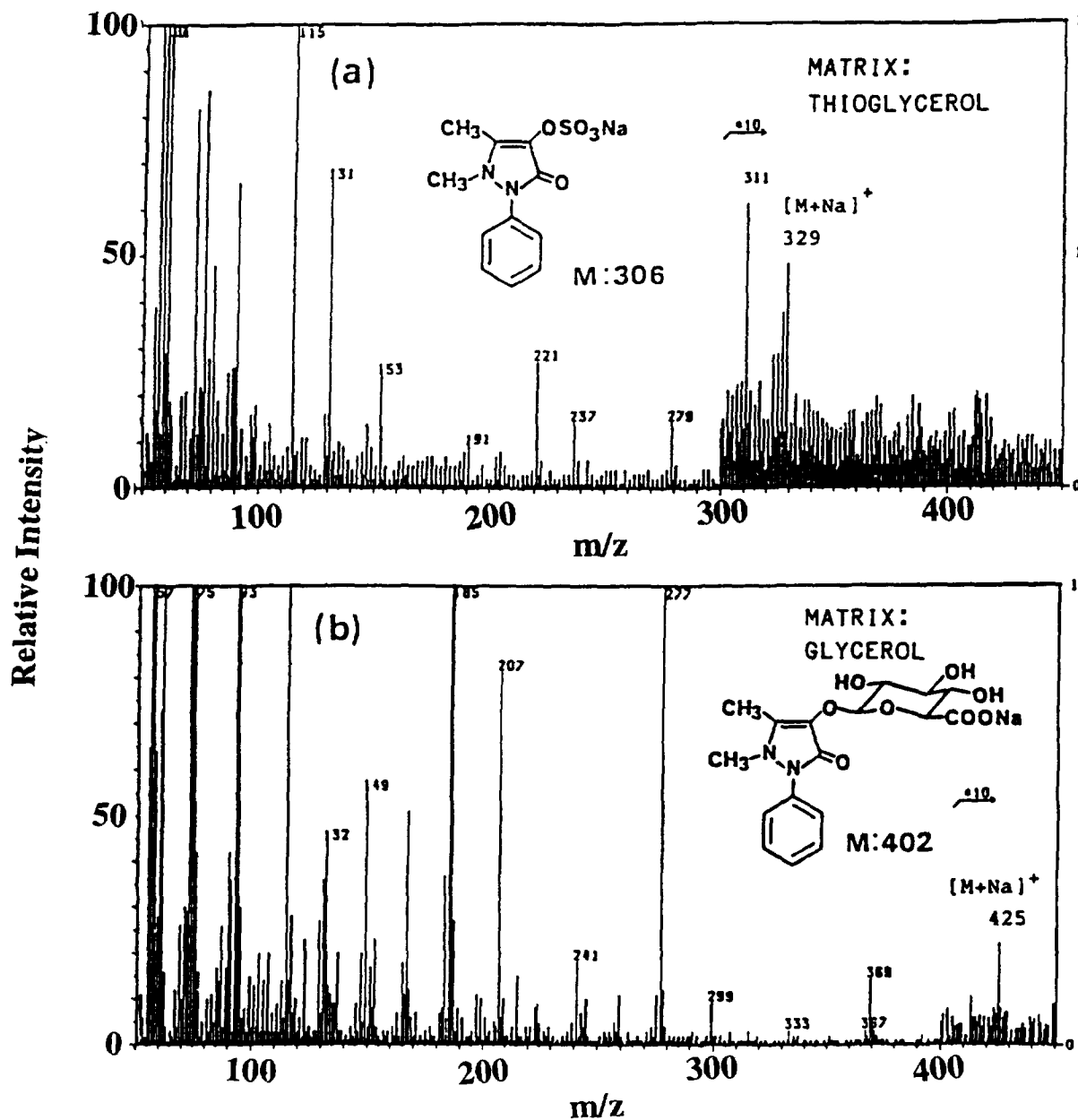


Fig. 7. TLC-SIMS of (a) 4-hydroxyantipyrene sulphate and (b) 4-hydroxyantipyrene glucuronide. Adapted from Ref. [38].

nide metabolites of *p*-nitrophenol, present in methanolic solutions of rat urine, was described. For these compounds negative-ion spectra were found to give better signal to noise ratios than those obtained in positive ion mode. Using thioglycerol as matrix the sulphate gave an $[M - H]^-$ ion at m/z 218 whilst the glucuronide gave

an $[M - H]^-$ ion at m/z 314 with a glycerol matrix (Figs. 8a and b). A limit of detection of 2 μg per spot was determined for both conjugates. The use of the technique for quantitative studies was also investigated (over the range 0 to 350 μg) using $^2\text{H}_4$ -labelled glucuronides and sulphates as internal standards. The method was

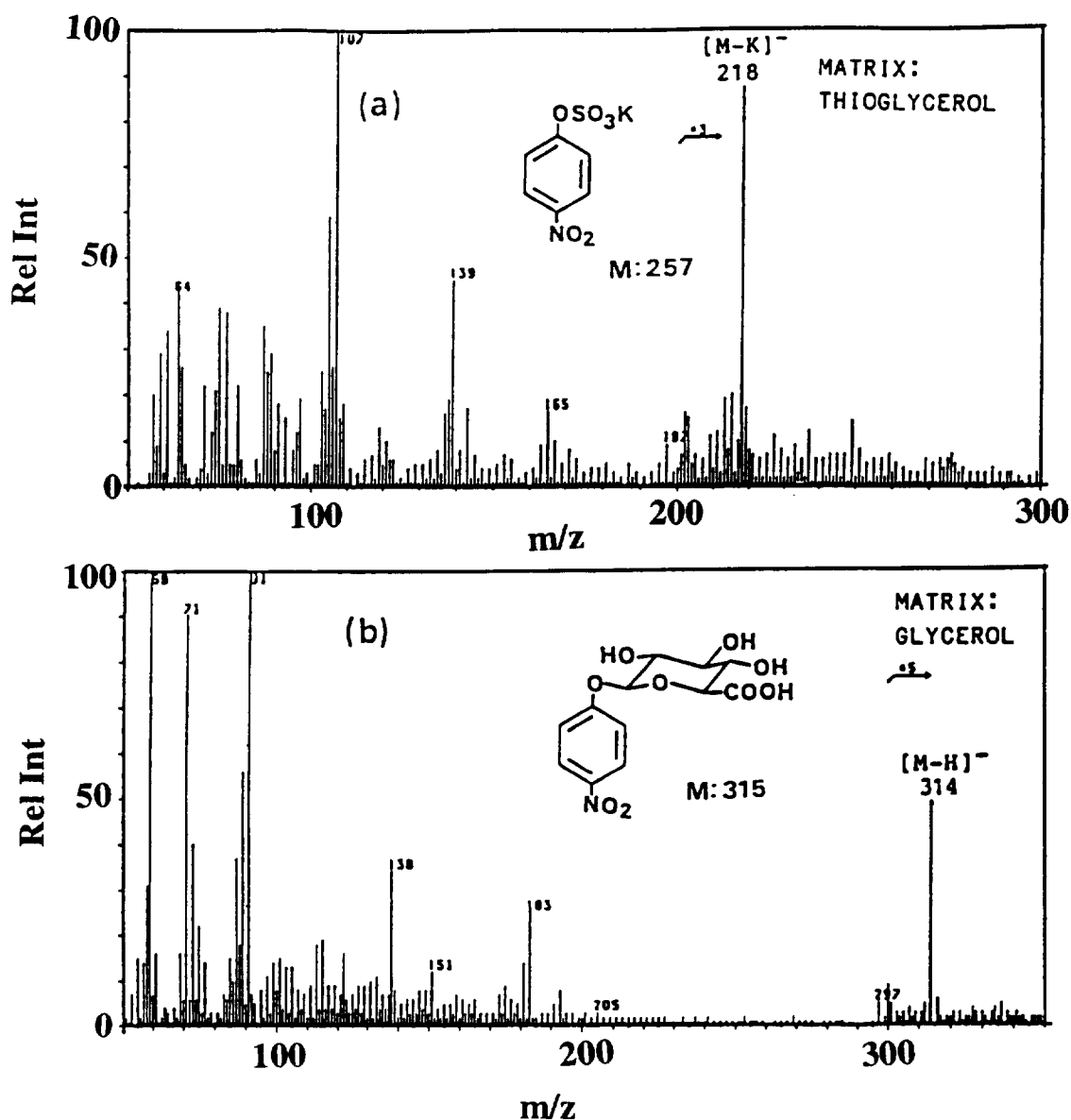


Fig. 8. TLC-SIMS of (a) *p*-nitrophenol sulphate and (b) *p*-nitrophenol glucuronide.

then compared with LC for the analysis of samples derived from rats with good agreement between the two techniques being demonstrated.

Scanning TLC-LSIMS was used in several applications to identify the metabolites of a range of compounds [23]. These included croconazole metabolites and their methylation/acetylation products, metabolites of the ex-

perimental drug S-145 and glucuronide and sulphate conjugates of a protoberberine.

In the case of croconazole glucuronide positive ion scanning TLC-LSIMS, with glycerol as matrix, was performed for the glucuronide metabolite and its methylated reaction product. These spectra were obtained from silica gel after chromatography with 1-butanol-acetic acid-water

(4:1:1, v/v/v). The same conditions were then used to analyse a mixture of the acetylated glucuronide and acetylated glucuronide methyl ester. In all cases good spectra were obtained with $[M + H]^+$ ions readily detected [23].

The preliminary examination of the metabolism of S-145 {(±)-5-(Z)-7-(3-*endo*-phenylsulphonylaminobicyclo[2.2.2]hept-2-*exo*-yl)heptenoic acid} was performed on C₈ glass-backed TLC plates with methanol–water–acetonitrile–acetic acid (1:1:0.5:0.005, v/v/v/v) as mobile phase [23]. This study showed that, although visualisation by UV-fluorescence indicated that spots were overlapping, and the positive and total ion mass chromatograms were not well resolved, metabolic information could still be obtained leading to the identification of a number of side chain oxidised products. Negative ion mass chromatograms and spectra also contributed to the identification of these oxidised metabolites as well as the taurine conjugate. Positive and negative ion mass spectra for the latter were obtained from aluminium-backed silica gel plates after chromatography in the system chloroform–methanol–acetic acid–water (13:10:1:3, v/v/v/v). The $[M + H]^+$ and $[M - H]^-$ at m/z 457 and 455 were readily visible together with those of sodium adducts and fragments showing the loss of SO₃ and the phenylsulphonyl residue.

In the last example given in this paper the authors showed spectra obtained for the sulphate and glucuronide conjugates of 9,10-dimethoxy-3-hydroxy-7-methyl-5,6,7,8,13,14-hexahydrodibenz[*c,g*]azecine, a protoberberine [23]. Good positive ion mass spectra were apparent from aluminium-backed silica gel (1-butanol–acetic acid–water, 4:1:1). Considerable enhancement of the mass chromatograms and the mass spectra was obtained after unspecified 'computer enhancement' of the data.

In a preliminary report the use of the Jeol TLC–FAB–MS interface in the analysis of a diverse range of compounds (atenolol, methylatropine, chlorpromazine, cimetidine, furosemide, muretin, meoclopramide, naloxone, renazpride and scopalamine) was described by Bolton et al. [39]. TLC was performed with silica gel plates and chloroform–methanol–ammonia

(10:4:1, v/v). Detection limits of between 0.5 and 5.0 μg of analyte applied to the plate for spiked dog urine were claimed for silica gel. The authors investigated a number of methods for applying the matrix concluding that dipping the plates in a mixture of methanol–glycerol (1:1) produced the best results. The conclusion of the study was that such interfaces would be "useful in drug development, notably in metabolism studies".

The identification of [¹⁴C]-paracetamol sulphate and glucuronide, present in rat urine following oral administration of paracetamol, was achieved by a combination of TLC on silica gel and FAB-MS [40]. Areas of radioactivity containing the radiolabelled metabolites were removed from the plate following chromatography, mixed with glycerol and subjected to negative ion FAB-MS. The sulphate was detected by ions at m/z 326 and 348 corresponding to the $[M - H]^-$ and the sodiated molecular ion respectively, whilst the glucuronide gave an $[M - H]^-$ at m/z 326.

5.2. Lipids

A simple and rapid method for the analysis of lipids using SIMS after separation on either aluminium or plastic-backed silica gel TLC plate has been reported [41]. Separation was achieved using a solvent system composed of chloroform–methanol–water (65:25:4, v/v). The analytes did not possess a chromophore and were therefore detected with iodine vapour or Coomassie blue. A modified Hitachi M-80 mass spectrometer was used to acquire spectra (xenon, 5 keV) from portions of the developed plates (maximum size 5 × 20 mm) attached to the probe tip via double sided tape. Approximately 1–2 μl of a solvent (usually methanol) and 2–5 μl of the matrix (glycerol or triethanolamine) were then over-spotted. The probe tip was manipulated manually in order to scan the plate, with spectra taken every 0.25 mm. Positive ion spectra were illustrated with clearly visible $[M + H]^+$ ions for a number of lipids including dipalmitoylphosphatidylcholine, sphingomyelin (from bovine

brain) lactosylceramide (human erythrocytes) and ganglioside GM₂ (from the brain of a patient with Tay-Sachs disease). Interestingly no interference was seen from either iodine, Coomassie blue or the masking tape used to attach the plate to the probe. Spectra could be obtained for approximately 1 µg of these lipids.

More recently the same group published further results on the TLC–SIMS of glycolipids obtained from extracts of the organs of patients suffering from Gaucher's, Tay-Sachs or Fabry's disease [42]. Silica HPTLC plates were used in conjunction with two solvent systems (chloroform–methanol–water, 65:25:4, and chloroform–methanol–water, 55:45:10). As before, separated lipids were detected by staining the developed plates with Coomassie blue, or with resorcinol. In the case of Gaucher's disease the identification of glucosylceramide ($[M - H]^-$ in negative ion mode, $[M + H]^+$ and $[M + Na]^+$ in positive ion mode) was observed in extracts of liver and spleen. The matrix used was triethanolamine. The authors claimed that each spot was readily identifiable with molecular ion species appearing in the range m/z 698 to 810. The range of molecular ions seen was due to variations in the composition of the ceramide portion of the analyte and corresponded to palmitate, behenate and lignocerate as fatty acid components. In the case of Fabry's disease TLC–LSIMS enabled the accumulation of globotriaosylceramide to be determined. Molecular ions in the range 1022 to 1134 were observed as a result of variations in the ceramide portion. Thus m/z 1022, 1078, 1106 and 1134 corresponded to the presence of either palmitate, arachidate, behenate or lignocerate as the fatty acid components, respectively. Some fragmentation as a result of cleavage of the glycosidic linkages was apparent but the intensity of these ions was low. With Tay-Sachs disease brain extracts were found to contain GM₂ ganglioside on the basis of the $[M + Na]^+$ observed at m/z 1406 and $[M - H]^-$ at m/z 1382 in positive and negative ion mode, respectively. Mass chromatograms were also shown, obtained as described above by moving segments of the plate through the ion

source manually in 0.25 mm increments and recording mass spectra at each step.

The latest application from this group to the analysis of lipids by TLC–LSIMS was to blood group A-active glycosphingolipids [43] following extraction from erythrocyte membranes. Chromatography for glycolipids was performed on silica gel with chloroform–methanol–water (65:25:4 and 60:35:8, v/v/v) whilst acetylated glycosphingolipids were separated with chloroform–methanol (95:5, v/v). Detection was by immunostaining with three major bands detected (designated A^a, A^b and A^c). Mass spectrometry was performed by cutting out the area of interest, fixing it to the probe tip and then applying a few microlitres of methanol and the SIMS matrix triethanolamine or glycerol. Data consistent with the presence of neolactohexaosylceramide (A^a) and neolactooctaosylceramide (A^b), with the fatty acid composition C24:0 and C24:1 and with the sphingoid base of d18:1 were obtained. Thus the component A^a detected by immunostaining gave an ion of m/z 1,686 corresponding to the $[M - H]^-$ of a molecule with a ceramide species of C24:0 fatty acid and d18 sphingene. Some fragments associated with cleavage of the glycosidic bonds were also clearly visible in the mass spectra. Similarly neolactooctaosylceramide gave an $[M - H]^-$ at m/z 2051. TLC–LSIMS of the peracetylated glycolipids as a means of enhancing sensitivity was also studied.

Busch and his co-workers [28] have studied the use of positive ion FAB for the determination of phospholipids from silica gel. A variety of phosphatidylcholines were separated on aluminium-backed silica gel using chloroform–methanol (30:70, v/v). The mixture analysed contained 1.5 µg of each of the distearoyl, dipalmitoyl, and dioctanoyl analytes. The mass spectra were obtained using a modified Extrel C-50 mass spectrometer with sorbitol as FAB matrix. The plate was scanned over 5 cm length in 2 min with mass chromatograms constructed on the basis of protonated molecular ions and common fragments. The use of the molecular ion allowed the separate detection of dipalmitoylphosphatidylcholine (m/z 734) and dioctanoylphosphatidylcholine

(m/z 510) which appeared as a single spot using iodine or fluorescence detection.

5.3. Bile acids

The first report of the use of TLC–LSIMS in the context of bile acid analysis was that of Tamura et al. [44] who used a motorised probe on to which the appropriate TLC track was attached. Subsequently Busch et al. described TLC–SIMS, with spatial imaging, of a number of bile acids, together with the identification and quantification of sodium taurocholate in an extract of dog bile [33,45]. Mixtures of standard bile acids were separated on aluminium-backed silica gel using iso-octane–ethyl acetate–acetic acid (10:10:2, v/v), whilst the dog bile extract was chromatographed on silica gel HPTLC plate with 1-butanol–acetic acid–water (40:10:10, v/v) as eluent [45]. Positive and negative ion mass spectrometry was performed using a modified Extrel C-50 quadrupole mass spectrometer using either LSIMS (caesium or gallium) or FAB (argon) depending upon the experiment. Mass spectra were acquired by cutting out the spot and affixing to the probe using double sided sticky tape followed by 10 μ l of the glycerol matrix. For imaging experiments a thin layer of molten sorbitol or threitol was applied to the plate with a microspatula and the plate positioned on the platform inside the spectrometer (the platform was heated to maintain the matrix near its melting point). Images were generally made using the gallium primary ion beam from a liquid ion gun mounted on the TLC–LSIMS instrument, except for the images acquired for the dog bile extract where the FAB gun was used. Spectra obtained from the plate were similar to those of standards directly from the probe, but with slightly higher backgrounds because less of the sample was available for ionisation and detection. In positive ion mode the $[M + H]^+$ ions were generally weak and fragment ions showing loss of water were dominant. In contrast in negative ion mode the $[M - H]^-$ ion predominated. If sodium was present (either added or already present in the silica of the plate) $[M +$

$Na]^+$ or $[M - Na]^-$ ions predominated depending upon the mode of ionisation. For TLC–LSIMS ca. 100 ng of material was sufficient to give a full negative ion mass spectrum, stable for 30 min, suggesting an actual detection limit in the region of 1 ng. For imaging the spots needed to contain ca. 1 μ g of the analytes. Good spatial imaging was demonstrated using cholic, chenodeoxycholic and lithocholic acids both in positive and negative ion mode. Plates were sometimes treated with sodium chloride after chromatography in order to obtain good images of lithocholic acid as its $[M + 2Na - H]^+$ ion (m/z 421). The dog bile extracts contained mainly sodium taurocholate (as either one or both of the dihydroxy taurine-conjugated isomers) which were observed at m/z 514 ($[M - Na]^-$). Sodium taurochenodeoxycholate/taurodeoxycholate was also detected (m/z 498). Quantitative analysis of the extracted sodium taurocholate was performed using the $[M - H]^-$ ion, giving a concentration of 38 mg/ml. The major drawback of the procedure was the lack of separation of the dihydroxy isomers; however, it was noted that the use of MS–MS techniques would overcome this problem.

The use of TLC–SIMS for the direct identification of bile acids following separation on silica gel was one of a number of applications shown by Chai et al. [46]. Separations were achieved using iso-octane–isopropyl ether–isopropanol (2:1:1:1, v/v) for free bile acids and *n*-butanol–acetic acid–water (7:1:2, v/v) for conjugates. The appropriate zone was cut from the plate and attached to the probe. An aliquot (2–3 μ l) of an extraction solvent (methanol) and matrix (glycerol) were applied, and mass spectra acquired using a VG Analytical ZAB mass spectrometer fitted with a caesium gun (25 keV). Spectra from lithocholic acid (125 pmol) glyco-chenodeoxycholic acid (100 pmol) and taurochenodeoxycholic acid (20 pmol) were shown, with $[M - H]^-$ ions at m/z 375, 448 and 498, respectively. A glycerol adduct (m/z 467) was also observed for lithocholic acid. The authors also claimed to have obtained an $[M - H]^-$ ion for 100 pmol of cholic acid which, they claimed, represented a

greater than 10 fold increase in sensitivity over previous reports.

5.4. Peptides

TLC–LSIMS of small peptides from silica gel was first briefly reported by Stanley et al. [37]. Examples of the use of the technique for obtaining mass spectra of glycylphenylalanine, bradykinin were described. Sorbitol was used as the matrix and it was found that for bradykinin the addition of a small amount of *p*-toluenesulphonic acid increased the abundance of the $[M + H]^+$ ions. In addition the authors described the concept of derivatisation of lysine residues with pyridinium salts to provide abundant secondary ions for positive LSIMS. Imaging analysis, using LSIMS, of bands of the peptides bradykinin and dynorphin 1–10 following the transfer of the analytes from polyacrylamide gel electropherograms to nitrocellulose by western blotting was reported by Stanley and Busch [47]. Direct imaging analysis of the gels themselves was not practicable because of the high concentrations of water (8–15%) which they contain however, the Western blotting technique proved to be a satisfactory approach to obtaining both SIMS spectra and for spatial imaging. For this work 20 μg of each of the peptides was applied to the gels for electrophoresis. Coomassie blue was used to locate the peptides following electrophoresis, but was found to interfere with subsequent mass spectrometry and a separate channel was therefore run in parallel to enable the location of the peptides to be determined. Spectra were obtained using glycerol for the matrix and that acquired for bradykinin showed a protonated molecular ion at m/z 1061, with the base peak in the spectrum seen a m/z 120 corresponding to phenylalanine. Other ions for various fragments were also observed. Imaging was successfully performed, with a matrix of sorbitol doped with *p*-toluenesulphonic acid, using the protonated molecular ions of dynorphin (1–10) (m/z 1236) and bradykinin.

Dunphy and Busch demonstrated imaging of peptides such as enkephalins and bradykinins by LSIMS from silica gel TLC plates [48]. However,

because of difficulties associated with obtaining good images following chromatography the bulk of the work described in this paper relates to results obtained by spotting the peptides (10 to 20 μg) at the appropriate R_f values. Current work was described as being focussed on obtaining better methods to extract and image peptides separated by TLC, including the use of alternative solvent matrices and thinner silica gel layers.

In-situ derivatisation with 2,4,6-trimethylpyrylium tetrafluoroborate has been demonstrated for the tripeptide Gly–Phe–Leu on silica gel with positive ion SIMS for detection [49]. The peptide was derivatised by suspending the TLC plate in a developing chamber 1 in. above a solution of 0.5 g of the pyrilium salt dissolved in 3 ml of water. The chamber was then heated at 90°C for 2 h. In order to deprotonate an intermediate species, thus causing ring opening and the formation of a vinylogous amide, the reagent solution was then alkalified with 0.5 ml of triethylamine and heated for a further 20 min at 80°C. In order to form the pyridinium derivative the same solution was then acidified to pH 3 with acetic acid and the chamber heated at 60°C for 30 min. For mass spectrometry sorbitol was used as matrix, giving spectra which showed the presence of the intact cation of the derivative (m/z 440) and fragment ions at m/z 281 and 326 (Fig. 9).

5.5. Porphyrins

Schurz and Busch used positive ion FAB-MS for the measurement of the mass spectra of a number of porphyrins directly from Empore silica gel TLC plates [50]. However, for this combination of analytes and TLC phases extraction into the FAB matrix using a special extraction device was found to give better results than direct spectrometry.

Chai et al. [46] examined the use of positive ion TLC–LSIMS for the analysis of urinary porphyrins (as their methyl esters) obtained from patients with congenital erythropoietic porphyria. Chromatography was carried out on aluminium-backed silica gel HPTLC plates using ethyl

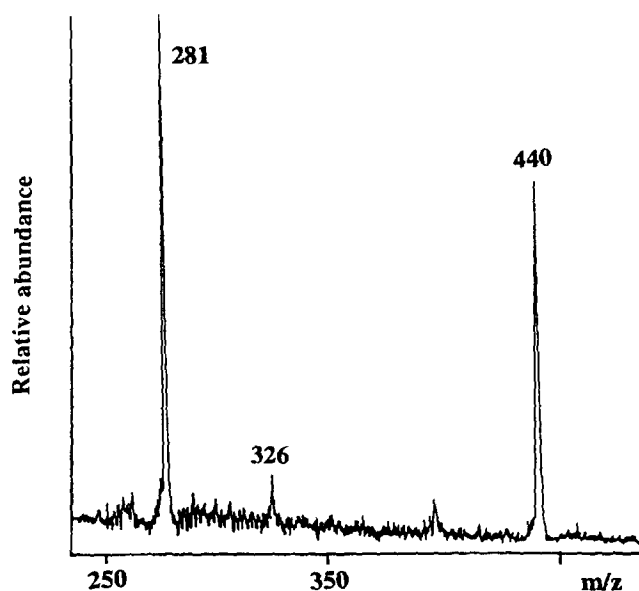


Fig. 9. Positive ion SIMS of Gly-Phe-Leu derivatised on the surface of a TLC plate with vapour from 2,4,6-trimethylpyrillium tetrafluoroborate.

acetate–dichloromethane–carbon tetrachloride (1:1:1, v/v). The appropriate bands were cut from the plates and attached to the probe. As described for their work with bile acids 2–3 μ l of chloroform–methanol (1:1) and matrix (thioglycerol) were then applied and mass spectra acquired using a VG Analytical ZAB 2E mass spectrometer with a caesium ion gun (35 keV). The presence of tetra- to octa-carboxylic acid porphyrin methyl esters was shown, together with a recently identified *meso*-hydroxyuroporphyrin I²² and other hydroxylated porphyrins at amounts corresponding to between 0.2 and 0.5 nmol. Both $[M+H]^+$ and $[M+Na]^+$ ions were detectable in the examples provided.

5.6. Oligosaccharides, polysaccharides and glycosides

Several examples of the valuable contribution that TLC–LSIMS can make in the characterisation of oligosaccharides have been described [51,52]. In the first study Stoll et al. [51] obtained a number of oligosaccharides (di- to hexasaccharide alditols) from human meconium glycopeptides. Following purification the indi-

vidual oligosaccharide alditol fractions were subjected to periodate oxidation and then coupled to dipalmitoylglycerophosphoethanolamine and subjected to HPTLC on silica gel with chloroform–methanol–water (130:50:9, v/v/v). Two lipid-linked fragments were obtained for each oligosaccharide alditol and these were well resolved from each other by HPTLC whilst mass spectrometry could be used to assign both the sequence and branching patterns of the oligosaccharides. Thus, after TLC the appropriate bands were cut out and placed on a standard stainless-steel probe for negative ion (caesium) LSIMS. A matrix of tetramethylurea–triethanolamine–*m*-nitrobenzoyl alcohol (2:2:1, v/v/v, 2 μ l) and chloroform–methanol–water (25:25:8, v/v/v 2 μ l) were placed on the silica prior to spectrometry. The masses of the neoglycolipids resulting from the coupling of the various oligosaccharides to the dipalmitoylglycerophosphoethanolamine ranged from m/z 997.6 up to 1727.9 a.m.u. The second application of this technique [52] described the use of essentially the same procedures to microsequence a number of minor tetra- to heptasaccharides.

The successful TLC–LSIMS of a number of oligosaccharides from silica gel HPTLC plates as their phosphatidylethanolamine dipalmitoyl (PPEADP) derivatives represented a third application of the technique by Chai et al. [46]. Following chromatography with chloroform–methanol–water (130:50:9, v/v) the derivatised oligosaccharides were located by spraying the plate with primulin. As before the plates were segmented and strips attached to the probe for application of a solvent (chloroform–methanol–water 25:25:8) and matrix (diethanolamine–tetramethyl urea–*m*-nitrobenzyl alcohol; 2:2:1, v/v). Mass spectra were acquired in negative ion mode. Surprisingly no interference from the detection reagent was observed in the spectra, although a 15% reduction in sensitivity was noted. The authors found that direct analysis of material on the plate was more sensitive than extracting the spot and then performing mass spectrometry (exemplified with lactose PPEADP). Storage of the plates at -20°C for a few weeks produced no detectable loss in sensitivity, whilst storage of an HPTLC plate with 1 nmol of lactose–PPEADP for 4 months under these conditions gave only a 30% reduction in sensitivity.

In a recent application Zhongping et al. [53] used TLC–FAB–MS for determination of the glycosyl sequence in glycosides employing gradual hydrolysis of the glycoside. TLC on silica gel was used to separate the hydrolysis products. Direct analysis of the spots from the TLC plate was then used to identify the products, and the glycosyl sequence was then deduced from the differences in the masses of the pseudomolecular ions of the original glycoside and the degradation products. Gypentoside A was isolated from *Gynostemma pentaphyllum* and incubated with cellulase for 24 h. The hydrolysate was then chromatographed using chloroform–ethyl acetate–methanol–water (15:40:22:10, v/v/v/v). In order to detect the products, half of the plate was charred with sulphuric acid. The remaining half of the separated bands were taken for spectrometry. Mass spectra were obtained using a VG ZAB–HS mass spectrometer fitted with an Ion Tech FAB gun (xenon). The TLC spot of

interest was touched with a stainless steel needle which had a small amount of glycerol on its tip. The silica gel was thus transferred to the probe tip on to which 1 μl of glycerol had been applied and, following the application of a small amount of a solution of sodium chloride in methanol, positive ion FAB–MS was performed in the usual way. All eight spots, including the parent compound gave $[\text{M} + \text{Na}]^{+}$ ions (gypentoside A = m/z 1131) enabling identification to be made of each of the separated components.

5.7. Dyes

A series of papers [24,54–56] have described the use of TLC–LSIMS for the identification of food dyes following chromatography on aluminium-backed plates. In the first of these articles the basic methodology was described, including a spot concentration (or condensation) technique, whereby the matrix (thioglycerol) was applied around the spot. After a short time the thioglycerol had diffused to the centre, concentrating the dye into a small area. Then a quantity of the ‘magic bullet’ matrix was applied to the concentrated zone which was then removed and attached to the LSIMS probe for mass spectrometry. Using this methodology sensitivities of 0.1 to 0.5 $\mu\text{g}/\text{spot}$ were achieved for the four dyes investigated, with $[\text{M} + \text{H}]^{+}$ and $[\text{M} + \text{Na}]^{+}$ ions readily observed. Sensitivity without spot condensation was 5 $\mu\text{g}/\text{spot}$ [54].

This preliminary work was followed by a more extensive investigation encompassing the 11 food dyes permitted for use in Japan [55]. Chromatography was performed on an aluminium-backed reversed-phase TLC plate prepared by impregnation of a silica TLC plate with liquid paraffin (10%). The solvent systems used included methanol–methyl ethyl ketone–10% aqueous sodium sulphate (1:1:3.5, v/v/v) and methanol–acetonitrile–10% aqueous sodium sulphate (3:3:10, v/v/v) depending upon the class of dyes. Positive ion FAB spectra were acquired on a Hitachi M-80B mass spectrometer with xenon (8 kV) and magic bullet as the matrix. Three classes of dye were investigated (‘xanthine, triphenylmethane and azo’) with sensitivities of 0.1 to 3

$\mu\text{g}/\text{spot}$ claimed. Satisfactory spectra were obtained for all of the compounds except for Indigo Carmine. An application was reported whereby the authors extracted and identified a dye from a 'foreign' candy which they identified as Azorubine, a dye not permitted in Japan. A further study, examining an improved spot condensation technique applied to acid red, amongst other analytes, with TLC–FAB–MS from glass-backed silica gel and C_{18} -bonded silica gel TLC plates was subsequently reported [24].

Most recently this group has reported further applications of TLC–FAB–MS to identify unlawful food dyes extracted from imported food samples [56]. Rather than reversed-phase chromatography on paraffin impregnated plates the dyes were separated on a C_{18} -bonded phase using either methanol–acetonitrile–5% aqueous sodium sulphate (3:3:10, v/v/v) or methanol–methyl ethyl ketone–5% aqueous sodium sulphate (1:1:1, v/v/v). Following chromatography a variation of the previously described sample condensation technique, involving applying a small amount of methanol around the sample spot on the developed TLC plate, was used. The plate was then placed on the TLC holder and the magic bullet FAB matrix applied. Mass spectrometry was then performed using a Jeol JMS-AX505W instrument. Xenon gas at 5 kV and an acceleration voltage of 3 kV was used to obtain FAB–MS data. Satisfactory spectra required at least 20 μg of material to be present in the spot unless the condensation technique was used to concentrate the analytes. With spot condensation the detection limit was reduced to between 0.03 and 5 μg , depending upon the compound. Thus xanthine dyes were the most sensitive, detectable at 0.03 to 1.0 μg per spot and triphenylmethane dyes could also be observed at 1.0 μg per spot. Applications to the detection of the unlawful dyes Azo Rubine, identified on the basis of ions at m/z 503 ($[\text{M} + \text{H}]^+$) and 525 ($[\text{M} + \text{Na}]^+$) in a candy sample extract, and Wool Green with ions at m/z 555 ($[\text{M} - \text{Na} + 2\text{H}]^+$), 577 ($[\text{M} + \text{H}]^+$) and 599 ($[\text{M} + \text{Na}]^+$) detected in powdered juice were described (Fig. 10).

Electrophoresis coupled with LSIMS has been demonstrated for the dye methylene blue sepa-

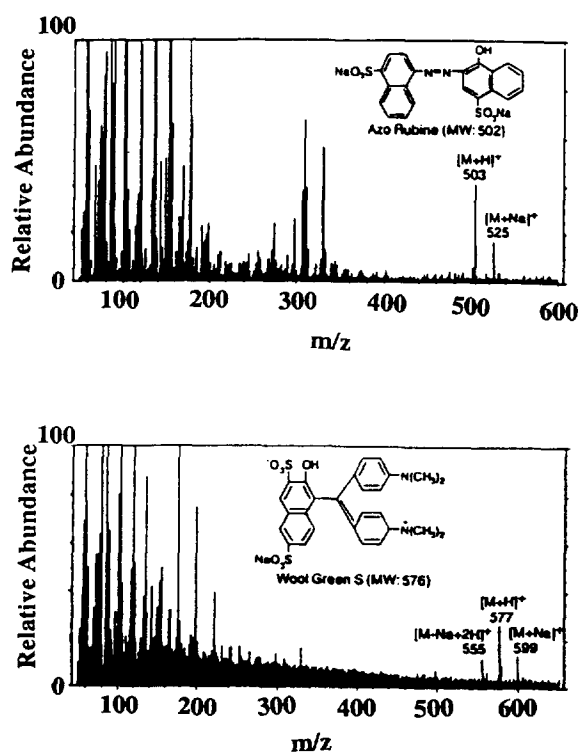


Fig. 10. TLC–FAB–MS of unknown dyes extracted from (top) candy and (bottom) powdered juice. Adapted from Ref. [56].

rated from other dyes and transferred onto a nitrocellulose support by capillary action. The ion for the protonated dye (m/z 284) was monitored to image the resulting spot [37]. The same laboratory has also reported the use of TLC–LSIMS for the spectrometry of two fluorescent dyes used in thin film sensor production [18]. The chief novel feature of this work was the development of a method for concentrating the spots of analyte prior to mass spectrometry. In this technique the TLC plate was cut in parallel to the developed track with 'pinking' shears to produce a regular patterned serrated edge. The plate was then turned at 90° to the original chromatographic separation and developed with a further solvent in order to concentrate the analyte at the tip of one of the wedges formed by the shears. This small area of the plate was cut off and attached to the probe tip of the mass spectrometer, matrix applied in the usual way,

and mass spectra obtained. The authors claimed that this simple procedure provided concentration factors of 10–20 fold.

5.8. Applications in the oil industry; surfactants and amine antioxidants

One of the first applications of FAB-MS to the direct identification of substances separated by HPTLC was from the analysis of surfactants used as oil additives and amines present in gas oils [57]. Separation of the surfactants was carried out on silica gel with toluene–methanol (80:20, v/v), whilst the amines were chromatographed using chloroform–ethanol (90:10, v/v). The surfactants were located on the plate by a combination of UV with fluorescence quenching and exposure to iodine vapour. Once located the spots were ringed using a pencil to provide a permanent record of their location and the iodine vapour was then driven off by heating the plates at 120°C for 15 min. Spectrometry was performed by covering the probe tip with double-sided adhesive tape, as described by Chang et al. [21], and then placing it in contact with silica scraped from the appropriate zone of the plate. The FAB matrix (thioglycerol) was then applied and analysis undertaken using the positive ion mode on a Kratos MS50 with argon as the reagent gas. One combination of surfactants investigated was a mixture of dodecylpyridinium chloride, poly(ethoxylated) nonylphenol and dodecylbenzene sodium sulphonate. FAB mass spectra were obtained for all of these components together with that of an ethoxylated aromatic amine present as a contaminant. Some complications with respect to the presence of both $(M + H)^+$ and $(M + Na)^+$ ions were noted in the case of the non-ionic surfactants.

Detection of the amines in gas oil samples was performed by selectively spraying part of the developed plate with a 'malonic acid' reagent (0.2 g malonic acid and 0.1 g of salicylaldehyde in 100 ml of ethanol). The amines present in the unsprayed portion of the plate were located by assuming that they had the same R_f values as those in the sprayed track. FAB-MS was then performed in the same way as for the surfactants

with sensitivities in the 20 $\mu\text{g/g}$ range readily achieved.

5.9. Organometallic compounds

Stanley et al. [37] briefly described the use of TLC–SIMS as a means of obtaining positive secondary-ion mass spectra of iron(III) acetylacetonate from silica gel with a crown ether phase transition matrix. The spectrum contained two large ions corresponding to $\text{Fe}(\text{acac})_2^+$ and $\text{Fe}(\text{acac})^+$ at m/z 254 and 155 respectively. The authors also provided a spatially resolved secondary ion chromatogram for overlapped spots of chromium acetylacetonate and iron acetylacetonate produced by monitoring the ions for $\text{Cr}(\text{acac})_2^+$ and $\text{Fe}(\text{acac})_2^+$.

5.10. Steroids

The use of negative TLC–FAB-MS for the confirmation of the identity of a number of ecdysteroids present in plant extracts has been described [58]. These compounds form a family of polar, polyhydroxylated, steroids which are important as insect developmental hormones. These compounds are also found in large numbers in certain species of plant. The extracts were analysed using normal-phase TLC on silica gel (chloroform–ethanol; 4:1, v/v). Removal of the band of interest and mixing with glycerol–DMSO, followed by FAB-MS enabled the unambiguous identification of a number of ecdysteroids present in plant extracts.

Brown and Busch used in-situ surface derivatisation with Girard's T reagent ([carboxymethyl]–trimethylammonium chloride hydrazide) to form derivatives of testosterone, cortisone, hydrocortisone and corticosterone [49]. The derivatisation involved suspending the silica gel plate over a solution of 0.5 g of Girard's reagent in 4 ml of glacial acetic acid–methanol (10:90, v/v) in a developing chamber and heating at 60°C overnight. The plate was then removed and placed directly on the sample platform of the spectrometer for imaging with sorbitol as the LSIMS matrix. The mass spectra obtained for testosterone showed the intact cat-

ion of the derivative (m/z 402) together with a number of fragments resulting from the loss of water (m/z 385) and trimethylamine (m/z 343). Similarly the intact cations of the derivatised hydrocortisone, cortisone and corticosterone were all detected (m/z 476, 474 and 460, respectively). The result obtained for a mixture of these analytes is shown in Fig. 11. The surface derivatisation approach used prevented the sort of spot diffusion sometimes experienced when spraying is used to prepare derivatives thus preserving resolution. An example of the spatially resolved mass spectral images obtained with these derivatives is given in Fig. 12.

The same research group [37] also briefly described positive and negative SIMS of the sulphate of androsterone following electrophoresis and electroblotting onto nitrocellulose.

5.11. Tetrodotoxin

Nagashima et al. [59] used TLC–MS to detect tetrodotoxin in extracts of two species of xanthid crab. Chromatography was performed on silica gel HPTLC plates with pyridine–ethyl acetate–

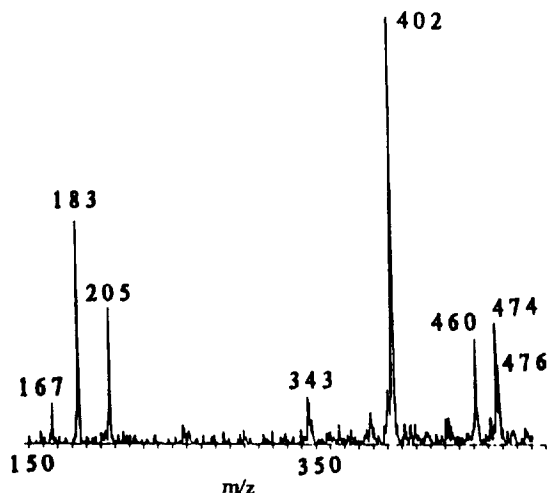


Fig. 11. Positive ion secondary ion mass spectrum of testosterone, cortisone, hydrocortisone and corticosterone derivatised on the surface of a TLC plate with vapour from Giraud's T reagent. Adapted from Ref. [49].

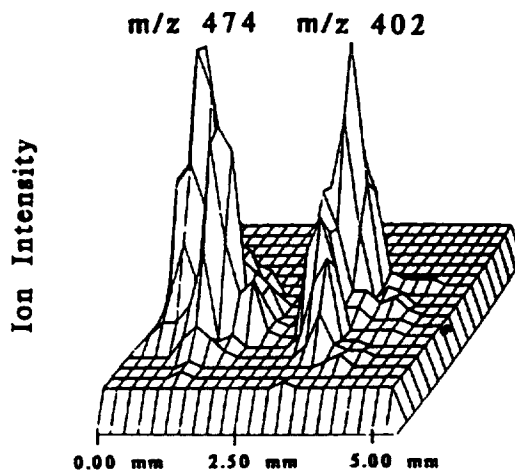


Fig. 12. Spatially resolved mass spectral image of cortisone (m/z 474) and testosterone (m/z 402) derivatised on the surface of a TLC plate with Giraud's T reagent. Adapted from Ref. [49].

acetic acid–water (15:5:3:4, v/v/v/v). The developed plates were then cut into 7×55 mm strips, coated with glycerol and attached to a plate holder for FAB–MS on a Jeol DX-303 mass spectrometer. Positive ion FAB mass spectra (xenon) were obtained for various standards, with tetrodotoxin giving an $[M + H]^+$ at m/z 320 and the $[M + H - H_2O]^+$ and $[M + Na - H_2O]^+$ and $[M + H + glycerol]^+$ ions detectable at m/z 302, 324, 342 and 412, respectively. Anhydrotetrodotoxin gave an $[M + H]^+$ ion at m/z 302 whilst tetrodonic acid gave an intense ion at m/z 320 and weak ions for $[M + Na]^+$ and $[M + H + glycerol]^+$ at m/z 342 and 412. When applied to the investigation of extracts the authors were able to identify tetrodotoxin and tetrodonic acid in extracts of *Zosimus aeneus* and tetrodotoxin in extracts of *Atergatis floridus*. The TLC–FAB–MS method was able to differentiate between tetrodotoxin and anhydrotetrodotoxin on the basis of molecular mass despite similar chromatographic properties. A limit of detection of $0.1 \mu\text{g}$ was claimed. In the same study the TLC separation was replaced by electrophoresis on cellulose acetate with the detection of tetrodotoxin shown.

6. TLC–laser desorption mass spectrometry (TLC–LDMS)

The use of laser desorption in connection with planar separations is of interest because of the possibility of sampling a very small area of a spot (a few μm). This property enables spot homogeneity to be determined (e.g., in the case of overlapping components), and also leaves the bulk of the material unchanged for further study. There is also no need for the use of matrices of the type required for FAB/LSIMS (e.g. glycerol).

There have been a number of examples of the use of LDMS applied to the analysis of compounds separated via TLC. Indeed, as described above, one of the early TLC–MS scanners used laser desorption combined with CI for the detection of PAHs, etc. [4,5]. Further applications of TLC–LDMS have included triphenylmethane dyes [60,61], general organics and surfactants in industrial fuel and oil formulations [62], PAHs and purine derivatives [63] and PTH amino acids, nucleosides and nucleotides, and cholic acid [9,28]. Some examples have also been provided for the TLC–LDMS of compounds separated on electropherograms [28].

The initial brief report by Hercules [60] on the use of laser desorption from TLC plates for dyes was followed by a more comprehensive description of the work [61]. Spectrometry was performed using a LAMMA-1000 laser microprobe (Nd:YAG laser, 265 nm) in combination with a time of flight (TOF) mass analyser. A total of seven triphenylmethane dyes, including gentian violet and brilliant green, were studied with separations performed on silica gel HPTLC plates (methyl ethyl ketone–acetic acid–isopropanol; 40:40:20, v/v/v). Appropriate areas of the TLC plate were mounted on a macro stage with precision X,Y,Z translators, allowing a maximum scanning range of $50 \times 50 \times 70$ mm. for spectrometry. The laser desorption of the analytes from the TLC plate was compared to that obtained for the same compounds from a normal zinc metal substrate. In these experiments it was found that more laser power was required to obtain positive ion spectra for the

dyes absorbed onto silica gel compared to zinc. However, the molecular cations of all the analytes were readily detectable, and background ions from the TLC plate, did not significantly interfere. The authors were able to show that, where the separation between individual pairs of dyes was not complete, mass spectrometry could be used to demonstrate this overlap. For all the dyes, except brilliant green, laser desorption from silica was associated with increased fragmentation due to the need for increased power. The need for a higher laser power density was attributed either to some sample charging, or to a stronger sample–substrate interaction of the analytes with silica compared to the metal substrate.

The same group also undertook a highly detailed study, using the same apparatus, on a chemically diverse group of compounds [63], where the TLC medium investigated was polyamide. As seen with silica gel a higher laser energy was required to desorb analytes from polyamide than from zinc metal. The effectiveness of the laser desorption process for all of these compounds from polyamide was amply demonstrated. However, examples of the technique following a chromatographic separation were confined to purine bases and PAHs. The purines used were guanine, adenine, purine and 3-methyladenine, with detection by positive ion spectrometry of the $[M + H]^+$ ion. A total of ca. 50 pg of each compound was applied to the plate. Adenine and guanosine showed similar chromatographic properties under the conditions used (dichloromethane as solvent) with overlapping spots, but these compounds were resolved on the basis of their different molecular ions (m/z 136 and 152, respectively). Purine and methyladenine gave $[M + H]^+$ ions at m/z 121 and 150 and by monitoring all of the protonated molecular ions the authors were able to construct a chromatogram of the separation.

For the PAHs (coronene, methylanthracene and rubrene), separated on polyamide using dichloromethane–methanol (40:60, v/v) the molecular ions were the most intense (m/z 300, 192 and 532, respectively) and these were used for determining the position of the compounds on

the plate following chromatography. In almost all cases interference from ions from the polyamide ($m/z < 150$) did not occur, and detection limits at the picogram level or below were claimed. The spatial resolution of the technique was claimed to be only limited by the diameter of the laser beam (ca. 5 μm).

Finney and Read [62] used laser microprobe analysis to detect a range of model compounds of the type encountered in industrial products such as fuels and oils. These included methoxybenzoic acid, *p*-aminobenzoic acid, cetylpyridinium bromide, sodium dodecyl sulphate and sulphate. Aluminium-backed silica gel TLC plates were used for chromatography (toluene–methanol; 4:1, v/v) with a LIMA-4 laser microprobe (Nd:YAG laser, 266 nm) mass spectrometer employed for analysis. Ionised species produced by the laser impact were mass analysed using a TOF mass spectrometer (both in positive and negative ion mode). The results obtained for *p*-aminobenzoic acid under a variety of conditions are shown in Fig. 13. The initial studies gave only weak spectra from samples on aluminium-backed silica gel TLC plates, but when in subsequent work the metal backing around the area of interest was exposed spectra were of better quality and higher intensity. The authors suggested that the technique was useful for confirmation of identity, but the identification of a “total unknown by this means alone would be tentative”.

In the laser desorption methods described by Busch et al. [9,28] a Nicolet FTMS-2000 Fourier transform mass spectrometer was used in conjunction with a Nd:YAG laser (at 266 or 1064 nm). In agreement with other studies using laser desorption the authors found that, for a range of compounds, a higher laser energy was required for compounds present on silica gel than on a stainless-steel substrate. Differences were also noted in the properties of C_8 and C_{18} bonded plates compared to silica. An investigation of the effect of the laser wavelength used was undertaken and revealed that PTH-amino acids, nucleosides and nucleotides could easily be detected using the 266 nm wavelength for desorption whilst bile acids, bile salts and peptides (at

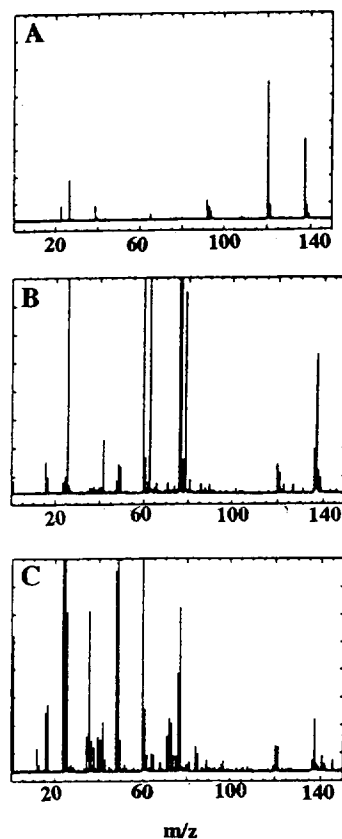


Fig. 13. Laser ionisation MS of *p*-aminobenzoic acid (A) reference standard (positive ions), (B) HPTLC plate origin (negative ions) and (C) after chromatography (negative ions). Adapted from Ref. [62].

the 5 μg level) could not. Laser desorption with the 1064 nm wavelength could also be used for nucleosides and nucleotides from C_{18} bonded material. An example of the use of the technique with electrophoretic gels was also discussed for 1-methylguanosine and deoxyadenosine. Negative ion mass spectra were obtained (266 nm) albeit with relatively high detection limits (10 μg).

7. TLC–tandem mass spectrometry (MS–MS)

Although, as described above, MS techniques are well established for the identification of organic compounds separated via planar chromatography there are drawbacks. The major prob-

lems with the simple TLC–MS approaches, such as those based on FAB, are the often significant contributions from the background and the relatively limited fragment ion information available from the spectra. This is not merely a limitation of TLC–MS, but can be a feature of all linked chromatography–MS systems. The use of tandem mass spectrometry (MS–MS), where two mass spectrometers are linked to form a single instrument, provides the basis for a solution to both the problem of background interference and lack of structural information when using TLC–MS with FAB. In MS–MS the molecular ion of the compound of interest can be selected using the first mass spectrometer (MS1), and thus separated from interfering background ions, and then have fragmentation induced in a collision cell containing a gas (usually He, Ar or Xe). These fragment ions are then focussed and detected by scanning with the second mass spectrometer (MS2). As these fragments correspond only to the ion selected using MS1 they provide a spectrum enabling the analyte to be characterised. Indeed such is the power of the MS–MS approach that for sometime it was thought that it could of itself replace chromatographic separations. This has proved to be somewhat optimistic as MS–MS cannot always differentiate between isomeric compounds. In addition, in complex matrices, the presence of components with a high surface activity can suppress components with a lower surface activity leading to non-detection. For these reasons the combination of a readily implemented technique such as TLC in combination with MS–MS affords advantages both to the chromatographer and the spectrometrist. To date, however, due no doubt in part to the limited availability of MS–MS instruments, relatively few groups have reported of the use of TLC–MS–MS. These applications are described below.

7.1. Ecdysteroids

As indicated earlier ecdysteroids form a large family of polyhydroxy steroids and TLC–MS–MS is of particular value in aiding the analyst to distinguish between isomeric compounds with

similar chromatographic properties. In the first example of the use of this technique [64] the ecdysteroids present in extracts of the plant *Silene otites* were separated on aluminium-backed silica gel HPTLC plates with chloroform–ethanol (4:1, v/v). The plates were then cut into strips and attached to a motorised probe. Glycerol, as the matrix, and DMSO (as co-solvent) were coated onto the silica gel and mass spectra obtained using a VG Analytical 70 SEQ tandem mass spectrometer by using LSIMS, in negative ion mode, with 30 keV caesium ions accelerated to 8 keV for mass analysis. The MS–MS data were obtained by taking the precursor $[M - H]^-$ ion formed for the individual ecdysteroids into a quadrupole collision cell, containing xenon at an indicated pressure of $1 \cdot 10^{-5}$ mbar. The resulting product ions were then determined on a second quadrupole, at unit mass resolution, prior to re-acceleration to 8 keV for detection using a post acceleration conversion dynode and photomultiplier system. Characteristic MS–MS spectra were obtained for the three ecdysteroids, 2–deoxyecdysone, 2-deoxy-20-hydroxyecdysone and 20-hydroxyecdysone, present in the extract.

In a subsequent study [65] samples from the plant *Silene nutans* and from the eggs of the desert locust, *Schistocerca gregaria*, were studied using essentially the same methodology. In this case, however, rather than use a special probe the appropriate band was removed from the plate and mulled with the matrix and co-solvent. LSIMS–MS was performed using a VG Analytical Autospec-Q tandem double focusing spectrometer. This combination of TLC and MS–MS enabled the unequivocal identification of polygodine B, 2-deoxecdysone and 20-hydroxyecdysone in the plant extract and 2-deoxyecdysone and ecdysone in the egg sample to be made.

7.2. Rhamnolipids

In another study in the natural product field HPTLC, combined with FAB-MS and FAB-MS–MS, was applied to the identification of a range of rhamnolipids isolated from extracts of un-

identified strains of *Pseudomonas* [66]. Rhamnolipids are composed of rhamnose or rhamnobiore linked to a β -hydroxy fatty acid (or a dimer), the actual composition of which depends on many factors including the strain of micro-organism, culture medium and growth conditions. Separations were performed using both silica gel (using chloroform–methanol–20% aqueous acetic acid 65:15:2, v/v/v) and C_8 -bonded silica (methanol–water–trifluoroacetic acid 90:10:0.25, v/v/v). Mass spectra were obtained using a Jeol MS–TLCPA10 TLC–FAB probe system and a Jeol JMS/SX102/102A tandem mass spectrometer of $B_1E_1 - B_2E_2$ geometry. For the separations performed on silica glycerol provided the FAB matrix, with *m*-nitrobenzyl alcohol used for the C_8 bonded phase. Xenon was used as the FAB reagent gas and helium as the collision gas. The study demonstrated that the C_8 bonded phase gave the best results for these compounds, with TLC–FAB enabling rapid and easy identification of the glycolipid components according to the type of polar (carbohydrate) headgroups on less than 100 ng of material. TLC–MS–MS allowed more detailed structural information to be obtained via CID of the $[M + Na]^+$ ions.

7.3. Drug metabolites

Another area where TLC–MS–MS has been used is in the separation and mass spectrometry of drug metabolites. Often the metabolism of such foreign compounds involves the attachment of a polar endogenous molecule to a suitable functional group on the molecule. This serves both to increase water solubility (to aid excretion) and to inactivate the compounds. Such metabolites are grouped together under the term conjugates and their high polarity and ionisable nature can make them an interesting challenge, both for chromatography and spectrometry. TLC–MS–MS has been studied as a means for the identification of conjugates such as paracetamol sulphate without the need for the recovery from the plate [67]. Following chromatography on silica the appropriate areas of the plate were located by fluorescence quenching, removed and

muller with glycerol which was used as the FAB matrix. The MS and MS–MS spectra were obtained using a VG Analytical ZAB-HSQ and tandem mass spectrometer of BEQ geometry. The FAB spectra were acquired using fast xenon atoms, whilst MS–MS data were acquired at a collision energy of 40 to 60 eV with argon as the collision gas (at an indicated pressure of $3.0 \cdot 10^{-6}$ mbar). From the TLC–FAB–MS experiments it was possible to detect the $[M + H]^+$ ions of all of the analytes, but in all cases there was some interference from the matrix, which could not completely be removed by background subtraction. When MS–MS was employed characteristic spectra were obtained which contained only ions from the compounds of interest; the spectra were essentially identical to those of the same substances obtained directly from the probe. Representative MS–MS spectra of paracetamol sulphate, phenolphthalein glucuronide and hippuric acid (the glycine conjugate of benzoic acid) are shown in Fig. 14.

TLC–MS–MS has also been performed on the metabolites of the drug antipyrine following enzymic hydrolysis of various glucuronide conjugates and solvent extraction from urine [68]. Essentially the same conditions for mass spectrometry were employed as for the previous study [67]. The metabolites present in the solvent extract were separated by HPTLC on silica gel using a single development in chloroform–methanol–trifluoroacetic acid (95:5:1, v/v). Then, following detection of the metabolites by UV quenching at 254 nm, the relevant bands were removed from the plates and muller with *m*-nitrobenzyl alcohol which was used as the FAB matrix. By this procedure good quality MS–MS spectra were obtained for antipyrine itself, the desmethyl metabolite norantipyrine and the two monohydroxylated metabolites, 3- and 4-hydroxyantipyrine. The use of TLC–MS–MS was particularly useful in the case of the hydroxylated compounds as these are isomeric, with an $[M + H]^+$ of 205 a.m.u. The use of TLC–FAB–MS alone therefore would not have enabled them to be distinguished from each other. However, these compounds were well resolved chromatographically and in addition, as

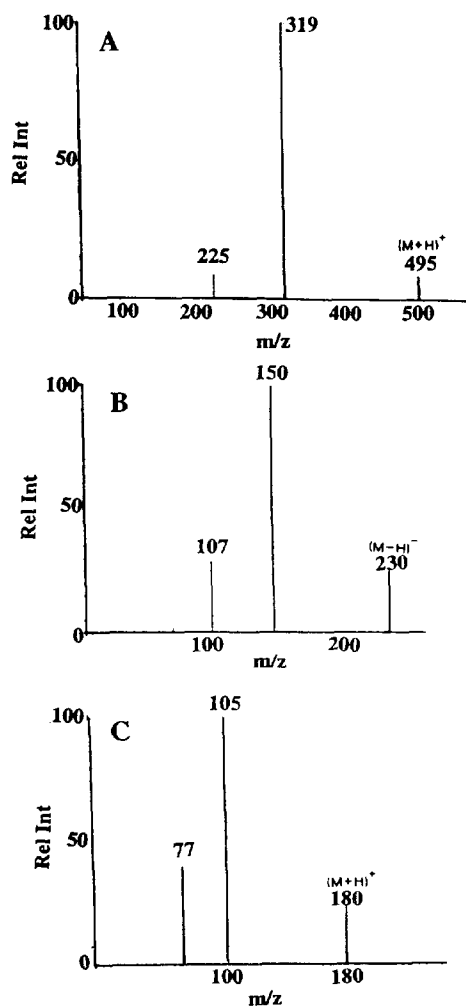


Fig. 14. Positive ion TLC-FAB-MS-MS of (A) phenolphthalein glucuronide, (B) paracetamol sulphate and (C) hippuric acid (for details see Ref. [67]).

shown in Fig. 15, the CID spectra for 3-hydroxyantipyrine (Fig. 15A) is quite different from that of the 4-hydroxylated metabolite (Fig. 15B). Potential interference from dibutyl phthalate, which was detected in the TLC-FAB-MS spectra, which also gave rise to an ion at m/z 205, was removed using the appropriate background subtraction.

These studies clearly demonstrate the potential of TLC-MS-MS in the drug metabolism field, both for the study of drug metabolism and for the confirmation of the identity of drugs of

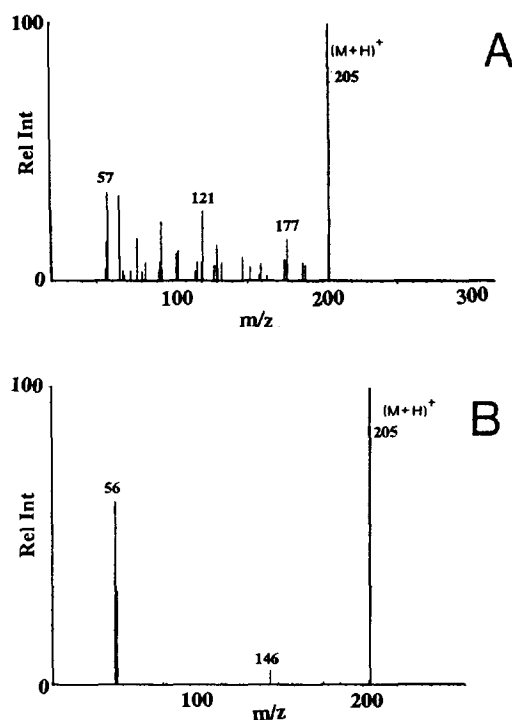


Fig. 15. TLC-FAB-MS-MS of (A) 3-hydroxyantipyrine and (B) 4-hydroxyantipyrine (for details see Ref. [68]).

abuse (many of which are initially screened for by TLC).

7.4. Polymer additives

Although the bulk of the applications in TLC-MS-MS use FAB or LSIMS as the initial ionising technique other methods of ionisation are equally applicable for suitable compounds. This has been demonstrated using electron impact (EI) ionisation on a mixture of polymer additives partially resolved by TLC on silica gel using an ethyl acetate-hexane solvent system [19]. The MS and MS-MS data were acquired using a VG Analytical ZAB-HSQ tandem mass spectrometer of BEqQ geometry. The EI data were acquired at an electron voltage of 70 eV and a trap current of 100 μ A. The ion source was maintained at 220°C. The appropriate zone of silica was removed from the plate and packed into the probe tip and the analytes were then slowly desorbed by increasing the temperature of the probe until

the ion current was sufficient for mass spectra to be obtained. The MS–MS data were acquired at a collision energy of between 20 and 60 eV, with argon as the collision gas (at an indicated pressure of $3.0 \cdot 10^{-6}$ mbar. Good MS–MS product ion spectra were obtained for the $[M - CH_3]^-$ ions at m/z 205 for Topanol O and m/z 515 for Irganox 1076, respectively. Representative TLC–EI-MS and TLC–EIMS–MS spectra are shown in Fig. 16A and B for Irganox 1076.

7.5. Nucleosides and bases

The use of TLC–FAB-MS–MS for the identification of nucleosides and bases following separation on silica gel and a variety of other bonded stationary phases has recently been described [69,70]. Under FAB-MS conditions nucleosides undergo facile decomposition to their corresponding bases, making it difficult to distinguish between them when both are present in a mixture. The use of TLC in this instance was therefore advantageous as it enabled the rapid separation of nucleosides and bases for subsequent identification. Separations of the analytes were investigated on silica gel and amino-, diol- and cyano-bonded HPTLC plates, using dichloromethane–methanol–ammonia based mobile phases, with the best overall result obtained on the amino phase. TLC–FAB-MS and TLC–FAB-MS–MS were obtained using a VG Analytical ZAB-HSQ tandem mass spectrometer of BEqQ geometry. Fast xenon atoms were used to acquire FAB mass spectra at an acceleration potential of 8 kV with the source temperature at ambient. MS–MS data were acquired at a collision energy of 40 eV with argon as the collision gas ($3.0 \cdot 10^{-6}$ bar). For spectrometry the band of interest was removed from the plate and mixed with either glycerol or *m*-nitrobenzyl alcohol, and then introduced into the ion source of the mass spectrometer using a standard FAB probe. Good MS–MS results were possible with all four TLC stationary phases investigated. For example, in the case of uridine, the double focusing mass FAB mass spectrum of the standard showed two major ions at m/z 113 and 245, the latter corresponding to the $[M + H]^+$ ion.

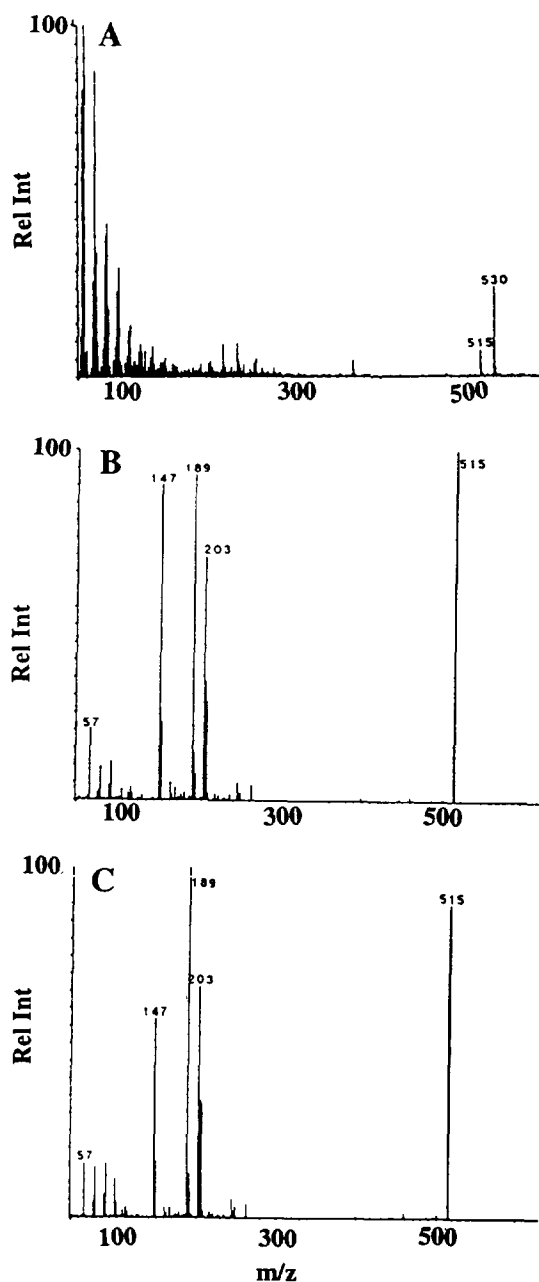


Fig. 16. (A) TLC–EIMS of Irganox 1076, (B) TLC–EIMS–MS of Irganox 1076 and (C) MS–MS of a standard of Irganox 1076 (for details see Ref [19]).

The ion at m/z 113 could have corresponded either to contamination of the uridine sample by uracil or to the fragmentation of uridine in the mass spectrometer. HPTLC–FAB-MS could

eliminate this possibility by separating the uridine from any contaminating uracil; however, spectra obtained in this way were subject to contamination by background derived from the plate and, in the case of glycerol, the FAB matrix. The FAB-MS-MS spectra obtained following HPTLC, with *m*-nitrobenzyl alcohol, were however, clear and unambiguous.

8. TLC-Fourier transform infrared spectrometry

The use of Fourier transform infrared spectrometry (FTIR) as a detection technique arises from the need to reliably identify components separated by TLC. FTIR has a highly discriminative capability and therefore in principle is a powerful identification method. Provided that reference spectra are available, almost all analytes, including structural isomers, can unambiguously be identified on the basis of their IR spectrum. When reference spectra are absent, valuable information about the molecular structure of the analysed compound may still be obtained by spectral interpretation.

Up to now the combination of TLC and FTIR has been approached in roughly two ways. In the first the FTIR measurement is performed in-situ, that is, the separated compounds are analysed directly on the TLC plate. Using this method spectral interferences can be expected as almost all TLC stationary phases have intense absorption bands in the mid-IR region. Therefore, the second, and usually more laborious, approach involves the transfer of analytes from the TLC plate to an IR-transparent substrate, prior to the FTIR measurement. Over the years, research has shown that in-situ as well as transfer methods can be effective and useful, each having specific advantages and limitations. Today, the TLC-FTIR methodology has advanced such that both in-situ and transfer interfaces are commercially available.

8.1. In-situ TLC-FTIR

The first in-situ FTIR detection of spots on a plate was demonstrated by Percival and Griffiths

[71]. A self prepared thin layer (100 mm) of adsorbent on an IR transparent AgCl support, allowed IR transmission measurement of dyes and amino acids at the 1–10 μg level. In later studies detection limits were improved by application of a mulling oil to the TLC plate to reduce IR scattering and by using programmed multiple development to concentrate sample spots [72,73].

Fuller and Griffiths [74] showed the feasibility of diffuse reflectance infrared detection (DRIFT) for the IR measurement of methylene blue on a silica plate in 1978, and since then DRIFT has become the most commonly used method for in-situ TLC-FTIR. Several studies have been performed to explore the potential of TLC-DRIFT analysis [75–78]. These studies, (extensively reviewed by Brown and Beauchemin, Jr. [79]), revealed that various conventional TLC phases, such as silica, alumina, cellulose and reversed-phase materials, can be used in combination with DRIFT, yielding minimum identifiable quantities (identification limits) down to about 1 μg .

The major difficulty encountered in using DRIFT as an in-situ detection method in TLC, is the strong absorption background of the adsorbent which causes serious interferences in particular spectral regions. For example, silica gel absorbs strongly in the regions from 3700 to 3100 cm^{-1} and 1600 to 800 cm^{-1} , obscuring possible analyte absorptions at these frequencies. Consequently, the DRIFT spectrum of a TLC spot is divided into parts where sensitivity is high and appropriate analyte information can be obtained, and parts where the signal-to-noise ratio is poor and only minimal information can be extracted.

To acquire useful IR spectra of analytes the background IR absorption of the adsorbent obviously has to be subtracted from the sample spot spectrum. To avoid an erroneous background correction, it is extremely important to record a representative blank plate spectrum that matches the background of the sample spot as closely as possible. Minor differences in e.g. the layer thickness and particle size of the adsorbent, the amount of residual developing solvents and the amount of adsorbed water, may have large

effects on the quality of the resulting analyte DRIFT spectrum. Therefore, the use of the same plate for both spot and background measurement is strongly recommended [78].

Due to interactions between the analyte and TLC stationary phase, small but significant changes in position and shape of absorption bands may occur in the IR spectrum of the compound under study. Hence, the TLC-DRIFT spectrum of a compound will differ from the spectrum obtained using conventional sample preparation methods (KBr powder or pellet). Nevertheless, the in-situ IR spectrum can still be used for spectral interpretation without any problem. However, the application of commercial spectral libraries for (automatic) analyte identification is limited, especially when discrimination is required between closely related compounds. More reliable identification would require reference libraries containing spectra of adsorbed species.

In all of these in-situ methods, the spots were localised on the TLC plate by UV light or by staining (using a second plate) prior to IR measurement. IR spectra were only recorded from selected spots and it was not possible to produce complete IR chromatograms. Glauninger et al. [80] developed an efficient diffuse reflectance unit on a computer controlled X–Y stage, which was in fact the first real IR densitometer for TLC. The entire TLC plate was placed in the unit and subsequently scanned by the IR beam while the stage was moving linearly. During the measurement of the TLC trace, interferograms were saved on disk and GC software used to generate individual spectra and spectral window- and/or Gram-Schmidt chromatograms. Positions of maximum substance concentration could be localised exactly and, if desired, remeasurement performed with high spectral resolution and co-addition of scans to gain both spectral quality and sensitivity. The software and the DRIFT unit, which used an 'off-axis' geometry to reduce specular reflectance, were specially optimised for TLC use, minimising the effect of the interfering adsorbent background as much as possible. Changes in the stationary phase, the support of the adsorbent and the layer thickness were reported to scarcely affect the quality of the

spectra. For an unambiguous identification, however, the use of a reference library consisting of TLC-FTIR spectra remains necessary.

Applications of the TLC-RIFT unit, which today is marketed by Bruker, were described by Kovar et al. [81] showing the separation and identification of drugs such as benzodiazepines (Fig. 17) and amphetamines. The method was used quantitatively and a comparison with UV- and GC methods was made. A thorough quantitative investigation of this direct TLC-FTIR method, was performed by Frey and co-workers

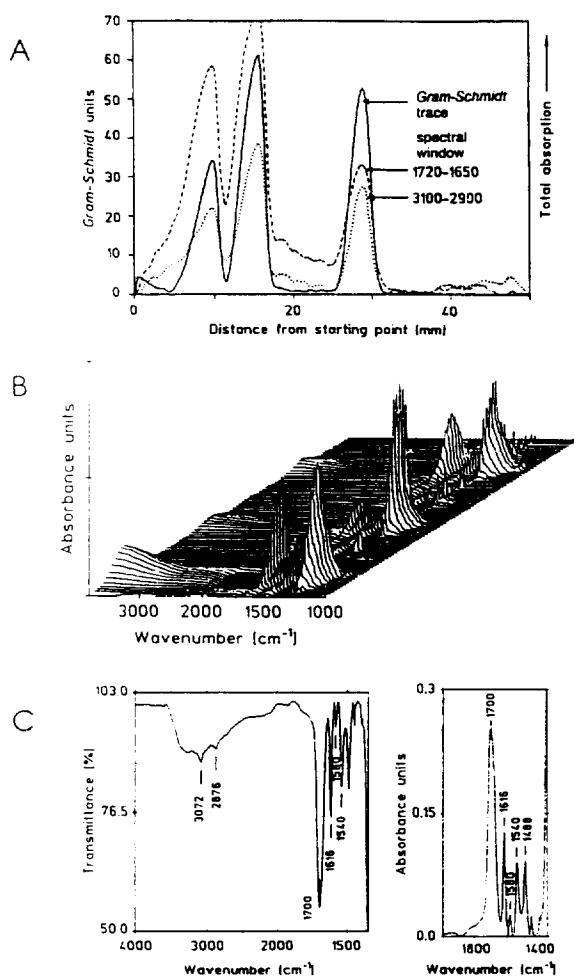


Fig. 17. TLC-FTIR chromatogram of a mixture of three benzodiazepines. (A) Gram-Schmidt and spectral window traces, (B) 3-D plot, (C) in-situ FTIR transmission- and absorption spectrum of nitrazepam. Adapted from Ref. [81].

[82]. Several important aspects including sources of error, precision, linearity, detection limits, analysis time and selectivity were discussed. In general, the quantitative characteristics were found to be satisfactory. Nevertheless, the limit of detection of the TLC–DRIFT system (ca. 100 ng) was about ten times higher than that of UV densitometry. Therefore, the strength of the method must be seen to lie in the identification of compounds, although the method can be particularly useful for the quantitative determination of substances lacking absorption in the UV region. This is clearly demonstrated by the TLC–FTIR determination of EDTA [81,83] in river water. EDTA, which is nearly UV inactive, was analysed as a cobalt(II) complex and chromatographed by automated multiple development (AMD). The in-situ DRIFT detection limit for EDTA was calculated to be 250 ng. Using solid phase extraction (SPE) for enrichment, 10–100 $\mu\text{g/l}$ EDTA could be analysed without the

derivatisation normally required with other methods. In general, use of AMD was shown to improve the DRIFT identification limit by one third compared to conventional TLC [84].

Recently, Danielson et al. reported on the use of a zirconium oxide TLC stationary phase in combination with DRIFT analysis [85,86]. Zirconia shows significantly higher IR reflectivity than silica or alumina resulting in only moderate background interferences. This is strikingly illustrated by the in-situ DRIFT spectrum of methyl red on both zirconia and silica (Fig. 18). When TLC was performed in zirconia-packed microchannels small analyte spots were obtained and subsequent use of microscopic DRIFT analysis yielded detection limits in the 1 to 10 ng range for several dyes [86]. Unfortunately, chromatographic use of zirconia is still limited and pre-coated plates are not commercially available.

An overview of substances analysed by in-situ TLC–FTIR, is shown in Table 1.

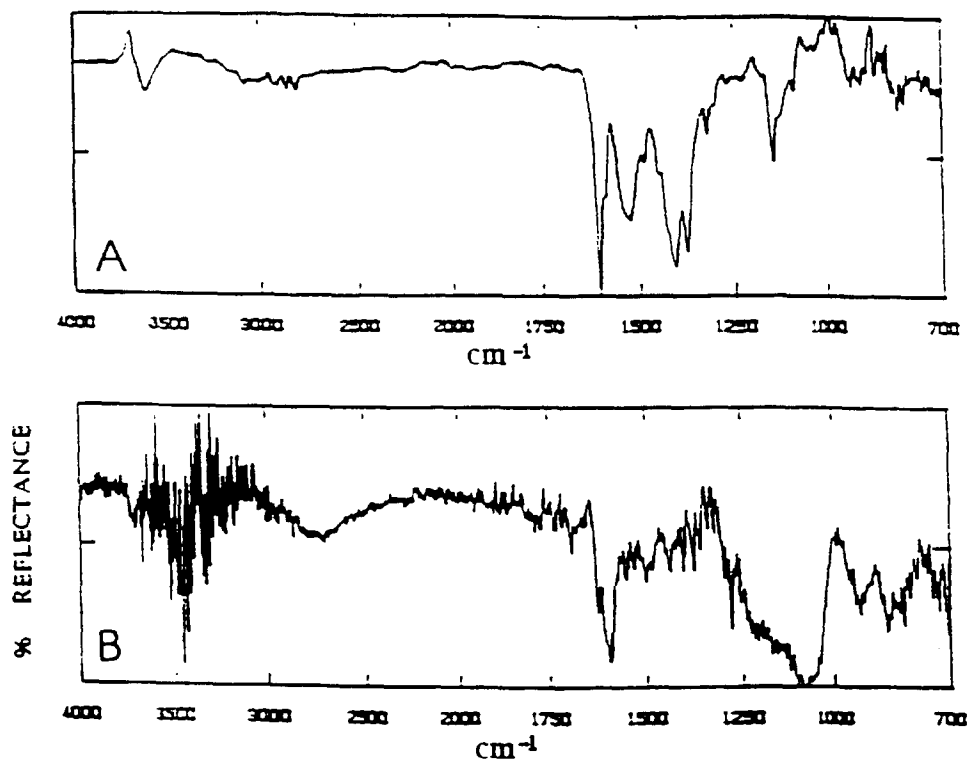


Fig. 18. In-situ TLC–FTIR spectrum of methyl red (10 μg) on (A) zirconia and (B) silica. From Ref. [85].

Table 1
Compounds analysed by in-situ TLC-FTIR^a

Compounds	Sorbent	FTIR mode	Detection limit (μg)	Ref.
Amaryllidaceae alkaloids	Sil	DRIFT	0.15	[88]
Amino acids	Sil, Alu	Transmission	0.5 ^b	[71]
Amphetamines	Sil	DRIFT	ND ^c	[81]
Analgesics	Cel	DRIFT	0.2	[76]
	Sil	DRIFT	0.1	[80]
	Sil	DRIFT	0.1 ^b	[82]
Benzodiazepines	Sil	DRIFT	0.5 ^b	[80]
	Sil	DRIFT	ND ^c	[81]
Diazonaphthoquinones	RP	DRIFT	0.4	[78]
Dyes	Sil, Alu	Transmission	0.2 ^b	[71]
	Alu	Transmission	0.01	[73]
	Sil	DRIFT	1 ^b	[74]
	Zir	DRIFT	0.3	[85]
	Zir	DRIFT	0.01	[86]
EDTA	Sil	DRIFT	0.25	[81,83]
PAHs	Zir	DRIFT	1	[86]
Pesticides	Alu	Transmission	10	[72]
	Alu	Transmission	0.01	[73]
Pharmaceuticals	Sil, Alu, Cel,	DRIFT	1 ^b	[75]
	RP			
Phenylureas	Sil, Alu, Cel,	DRIFT	50 ^b	[76]
	RP			
Phthalates	Sil, RP	DRIFT	1	[77]
Surfactants	Sil	DRIFT	50 ^b	[87]
Xanthines	NH ₂	DRIFT	ND ^c	[89]

^a Abbreviations: Sil = silica; Alu = alumina; Cel = cellulose; RP = reversed-phase; Zir = zirconia; NH₂ = amino phase; ND = not determinable.

^b Estimated on the basis of reported data.

^c Analysed amount of substance not stated.

8.2. Transfer TLC-FTIR

Transfer of analytes from the TLC plate to a proper medium is probably the most readily implemented way to couple TLC with a spectrometric technique. Standard, non-dedicated spectrometers can be used and scraping off a TLC spot and dissolving the analyte in a suitable solvent does not require special equipment, so that the coupling can be accomplished rather simply. On the other hand, as discussed for TLC-MS, transfer methods are usually time-consuming and they have a potential for loss, decomposition and/or contamination of the sam-

ple. Furthermore, it is often difficult to completely maintain chromatographic resolution during a transfer process.

Over the years, numerous transfer methods for the combination of TLC and IR detection have been described [76,77,90–96]. Today, the main reason for using sample transfer in TLC-FTIR, is to avoid the strong IR-absorbance of the TLC adsorbent by extracting the analytes via a solvent to an IR transparent pellet or powder. This makes it possible to measure full spectra at a reasonable sensitivity using conventional FTIR transmission or diffuse reflection detection. Fuller and Griffiths [91] showed that following a

transfer procedure involving scraping off, extraction, centrifugation, filtration and concentration, good-quality DRIFT spectra could be obtained of small amounts (100 ng) of dyes separated by TLC. In an attempt to simplify the sample preparation, during the last ten years, transfer methods have been developed for TLC-FTIR, in which the analyte is eluted from the plate without scraping off the adsorbent.

Issaq [97] applied the Eluchrom system (Camag) to automatically and quantitatively elute analyte spots from a silica plate using only 150 μ l of methanol per spot. The methanol was deposited onto KBr powder from which, after solvent evaporation, a pellet was produced. Using dispersive micro-IR-spectroscopy, Issaq obtained spectra of PAHs at the 5 μ g level.

To transfer a compound from an aluminum-backed TLC plate into a KCl pellet, Chalmers and co-workers [98] cut out the TLC spot and put it together with the pellet and 2 ml chloroform into a sample tube. During slow evaporation of the solvent to dryness, the analyte is collected in the pellet and subsequently its DRIFT spectrum is recorded. Applicability of the method was shown for the determination of antioxidant additives in polypropylene.

Iwaoka et al. [99] developed a method which involved the classical 'wick-stick' technique [100] for sample transfer. After normal TLC development KBr micro pyramids (80 mg) were pressed onto the side of the TLC plate at locations corresponding with the analyte spots. With a second development perpendicular to the first one, the analytes were eluted into the pyramids, which were then dried and pressed into pellets suitable for FTIR transmission measurements.

A sample transfer method which allows direct transfer of the TLC chromatogram into a series of cups filled with IR-transmitting glass powder, has been introduced by Shafer and co-workers [101]. Each cup is in contact with the TLC plate via a bundle of glass fibers and dichloromethane is used to perpendicularly extract the separated compounds into the cups. After transfer and solvent evaporation the strip with sample cups is placed onto a translational stage in the FTIR spectrometer and DRIFT spectra recorded from

each cup. This methodology allows a (discontinuous) IR chromatogram to be reconstructed. The use of the method was illustrated by the analysis of dyes and quinones (Fig. 19). Based on this transfer principle, a sophisticated TLC-FTIR accessory was commercially introduced by Analect (Laser Precision Analytical) in 1986. TeVrucht et al. [102,103] used this system for the TLC-FTIR analysis of coal extracts and Herman and Shafer [104] showed its potential in bioanalysis.

Shimadzu introduced an accessory for the transfer of TLC spots to a layer of KBr powder [105–107]. The technique involves the use of TLC sheets with a liquid-permeable support like the Empore TLC sheet. After ordinary TLC development, the sheet is placed into a holder and a thin layer of KBr powder is applied on the upper side. The bottom of the sheet is put in close contact with a plate of fritted-glass, which is wetted with a transfer solvent (i.e. methanol). The solvent ascends through the entire Empore sheet, eluting the analytes into the KBr layer. After drying, the holder is placed into a DRIFT attachment on a stage and a continuous reconstructed IR chromatogram can be obtained, while moving the stage at a constant rate. Provided that the transfer is accelerated by blowing hot air onto the powder, almost no loss in chromatographic resolution was observed, as shown by comparison of the IR chromatogram with the UV chromatogram recorded before transfer (Fig. 20). The identification limit is reported to be 2 μ g [107].

Chen and Smart [108] have described a TLC-FTIR method in which the analytes were thermally desorbed from silica gel. TLC spots were scraped off and measured using a commercial thermogravimetric analyzer-FTIR combination yielding vapor-phase transmission spectra. Qualitative and quantitative aspects were studied, and detection limits of about 1 μ g reported. Obviously, this methodology can only be used for substances and TLC adsorbents that are thermally stable.

An overview of analytes and transfer methods used in TLC-FTIR for the last fifteen years, is given in Table 2.

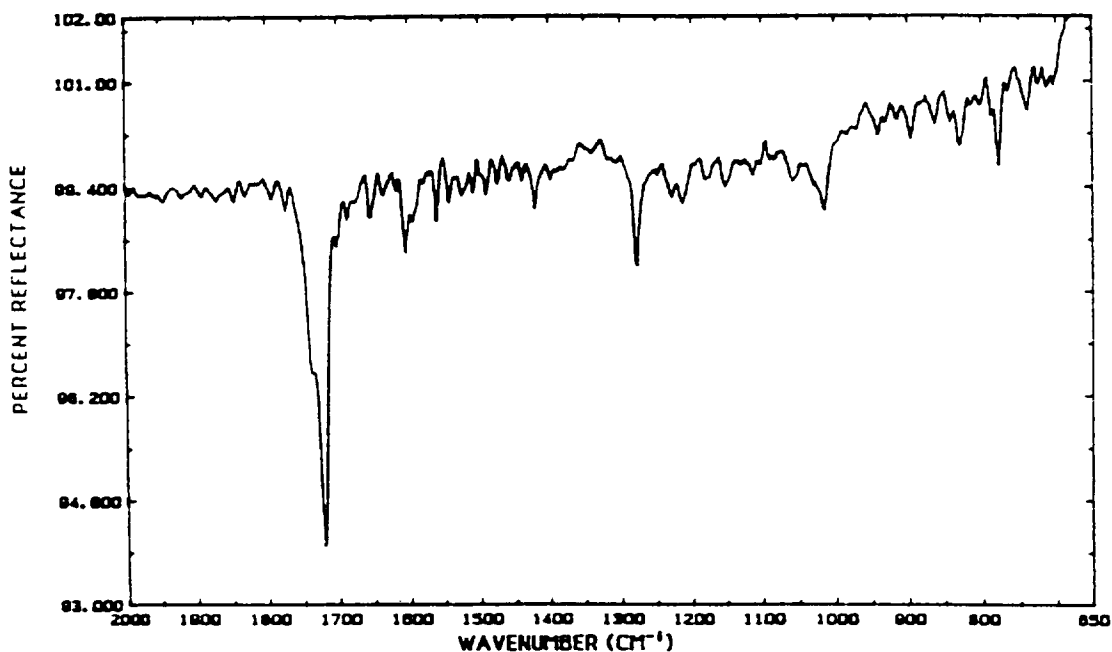


Fig. 19. Transfer TLC–FTIR of 1 μg acenaphthenequinone. From Ref. [101].

8.3. Near infrared spectroscopy and FTIR photoacoustic spectroscopy

This section will detail the few reported applications of near-infrared spectroscopy (NIR) and Fourier transform infrared photoacoustic spectroscopy (FTIR-PAS) as detection methods for TLC. NIR absorptions are overtones and combination bands whose fundamentals are in the mid-IR range. The major contributions to NIR spectrum originate from the vibrational stretching activity of C–H, O–H, S–H and N–H bonds and their combination with other vibrations. Nearly all analytes have a characteristic NIR spectrum, however, the spectral interpretation in terms of molecular structure is usually rather complex. In practice, NIR methods are dependent on calibration procedures involving training sets and chemometrics and often a calibration is required for each constituent [109]. A small number of research groups have studied the applicability of NIR as an in-situ detection tool in TLC. The main reason for this is that adsorbents such as silica gel have no strong absorption in the NIR

region, so background interferences are expected to be small. Furthermore, NIRS can be suitable for analysis of compounds lacking UV absorption, so that detection is possible without prior derivatisation.

The feasibility of dispersive NIR in its reflectance mode for TLC was studied by Ciurczak and co-workers [110–112] both for quantitative and qualitative analysis. Improved design of illuminating and collecting optics enlarged the initial signal-to-noise ratio resulting in detection limits of about 1 μg . The system was used for the quantification of several pharmaceuticals and linear calibration plots up to 200 μg were obtained [110]. Spectral subtraction of the silica background-signal remains a requirement of this technique. For qualitative determinations second-derivative spectra were used because they showed richer spectral detail and were more susceptible to small structural changes. Since the NIR spectra are affected by the analyte–adsorbent interactions, it was necessary to create a spectral library of adsorbed species. Using a spectral matching program several amino acids

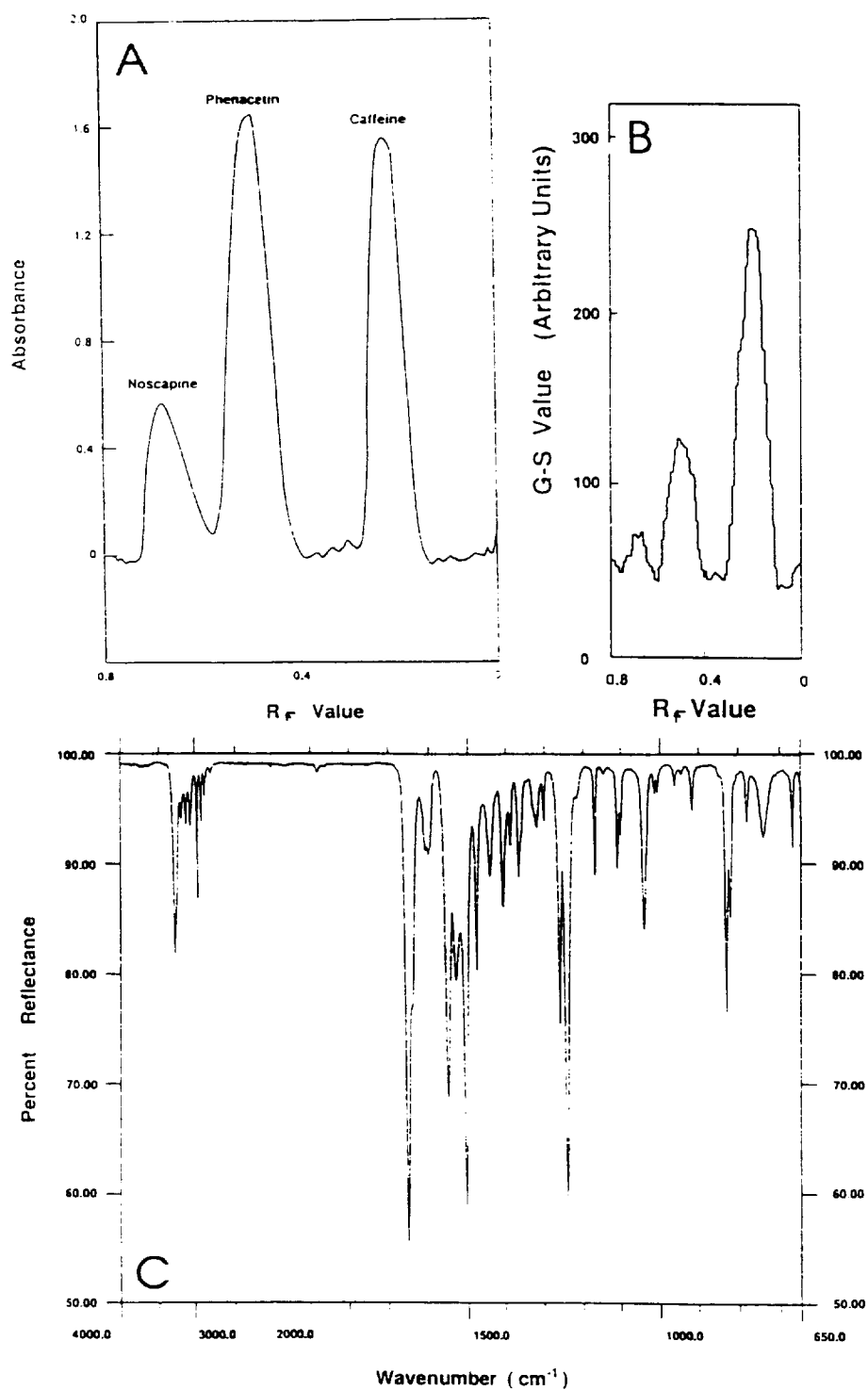


Fig. 20. TLC separation of a pharmaceutical mixture. (A) UV trace, (B) reconstructed IR chromatogram after transfer, (C) DRIFT spectrum of phenacetine after transfer. Adapted from Ref. [106].

Table 2
Compounds analysed by transfer TLC-FTIR^a

Compounds	Transfer method ^b	FTIR mode	Detection limit (μg)	Ref.
Amino acids	Shimadzu	DRIFT	2 ^c	[107]
Benzodiazepines	Scrape + Wick-Stick	Transmission	20 ^c	[93]
Coal extracts	Chromallect	DRIFT	ND ^d	[102,103]
Corticosteroids	Chromallect	DRIFT	2 ^c	[104]
Dyes	Scrape + extr.	DRIFT	0.01 ^c	[91]
	Scrape + Wick-Stick	Transmission	ND ^d	[92]
	In-situ extr. + Wick-Stick	Transmission	1 ^c	[99]
	Chromallect	DRIFT	1 ^c	[101]
Esters and ketones	TGA-FTIR	Transmission (vapour)	1	[108]
PAHs	Eluchrom	Transmission	1 ^c	[97]
Pharmaceuticals	Shimadzu	DRIFT	0.2 ^c	[105,106]
Phenols	Scrape + extr.	Transmission	2 ^c	[76,95]
Phthalates	Scrape + extr.	DRIFT	0.05 ^c	[77]
Phospholipids	Shimadzu	DRIFT	0.1 ^c	[104]
Polymer additives	Scrape + Wick-Stick	DRIFT	1 ^c	[94]
	In-situ extr.	DRIFT	10	[98]
Water constituents ^e	Scrape + extr.	Transmission	5	[96]

^a Abbreviations: ND = not determinable; PAHs = polyaromatic hydrocarbons.

^b Transfer methods: Shimadzu = using TLC-FTIR accessory of Shimadzu; Chromallect = using TLC-FTIR accessory of Analect; Scrape + extr. = spot scrape off followed by solvent extraction and evaporation on KBr; Scrape + Wick-Stick = spot scrape off followed by Wick-Stick technique; In-situ extr. + Wick-Stick = in-situ spot extraction followed by Wick-Stick technique; TGA-FTIR = using thermogravimetric FTIR analyzer; Eluchrom = using Eluchrom system (Camag); In-situ extr. = in-situ spot extraction followed by evaporation on KCl.

^c Estimated on the basis of reported data.

^d Analysed amount of substance not stated.

^e Constituents of surface water, waste water, sludge and sediments.

[111] and drugs [112] were identified on the basis of their TLC-NIR spectra. The possibility of differentiating between isomers was also highlighted by these workers.

Yamamoto et al. [113] used a microscope linked with a FT-NIR spectrometer to analyse TLC spots in-situ. The NIR spectra were obtained by measuring the diffuse transmission of the plate. Phospholipids that lack UV chromophores, were used to evaluate the method. By translating the microscope stage, NIR chromatograms could be constructed. Linear calibration curves (1.25–10 μg) and a detection limit of ca. 1 μg were obtained using second-derivative spectra. Qualitative analysis using the NIR spectra was not discussed.

Fong and Hieftje [114] demonstrated an in-situ TLC-NIRS technique using a diffuse transmitting geometry and a set of 18 interference filters

for wavelength selection. Non-uniformity of plates and varying amounts of adsorbed water were found to be the major complications, but could be alleviated by using proper reference measurements and chemometrics. After scanning the TLC plate using all interference filters, a NIR chromatogram could be reconstructed. For sugars the detection limit was near to 1 μg . Although relatively cheap, the proposed method was very time consuming and did not provide spectra, and is therefore not suitable for identification purposes.

Photoacoustic spectrometry (PAS) is based on the phenomenon that modulated light impinging on a solid, can produce an acoustic signal. In this technique, a solid sample is placed inside a closed cell containing an inert gas (i.e. helium) and a sensitive microphone. Upon illumination by the modulating source, light absorbed by the

sample is converted into heat. The periodic heat transfer to the gas causes pressure fluctuations which are detected by the microphone. The magnitude of the acoustic signal corresponds to the amount of light absorbed by the sample and consequently, a photoacoustic spectrum resembles an optical absorption spectrum. Qualitative and quantitative information on TLC samples has been obtained by dispersive and laser PAS at UV and visible wavelengths [115–118]. The interferometer in an FTIR spectrometer modulates the light of the source with a wavelength-dependent frequency. Therefore, in combination with a PAS cell, an FTIR spectrometer can yield photoacoustic IR spectra, which obviously can be applied for identification purposes.

Lloyd and co-workers [119] explored the use of FTIR–PAS for in-situ detection in TLC, analysing samples spotted on excised TLC disks in a PAS cell. The measurements were strongly disturbed by the background signal of the silica, resulting in poor detection performance. In a feasibility study using improved instrumentation, White [120] obtained better results. Samples deposited on silica powder were analysed in a PAS cell which was thoroughly purged with helium to prevent gas-phase interferences. After subtraction of the silica contributions to the signal, interpretable IR-spectra were obtained from analgesics at the 50 μg level. Detection limits were reported to be 1 μg . Marked differences between TLC–FTIR–PAS spectra and KBr transmission spectra were observed for polar compounds.

9. TLC–Raman spectroscopy

Raman spectroscopy (RS) can provide a useful means of identifying compounds separated by TLC. Like IR absorptions, Raman bands derive from the fundamental vibrational modes of the molecule, yielding an unique spectrum which allows analyte identification. Structural information can be extracted from Raman spectra in an IR-like fashion using functional group frequencies. The Raman effect can be described as the inelastic scattering of light by matter. This scat-

ter phenomenon is inherently weak, typically 10^{-6} % of the incident exciting radiation. In fact, modern RS relies on the availability of lasers as intense and stable light sources.

The main reason that RS has been considered as a tool for in-situ vibrational analysis of TLC spots is that TLC adsorbents such as silica give weak Raman spectra. Thus, compared to in-situ IR detection, low background interferences can be expected. However, the overall Raman sensitivity is usually rather poor and only few studies on TLC–RS have been reported. Recently, some improvements in TLC–RS performance have been accomplished using NIR-FT-Raman spectrometers. Major gains in Raman sensitivity can, however, be achieved by application of resonance Raman spectroscopy and surface enhanced Raman spectroscopy.

9.1. Normal RS

Adams and Gardner [121] were probably the first to study in-situ RS of TLC spots. Using an Ar-ion laser (488.0 nm) for excitation, a detection limit of about 50 μg was obtained in the most favourable case. Later TLC–RS studies [122–124] showed similar results, although detection limits could be improved applying micro-RS [124]. With visible-laser excitation, the observed Raman background is usually weak, but often TLC plates show a considerable fluorescence background, which disturbs the analyte Raman spectrum. Moreover, visible excitation is prone to induce sample fluorescence and/or sample degradation.

The recent introduction of NIR exciting FT-Raman spectrometers [125] has prompted some research groups to re-assess RS as an in-situ detection method for TLC. Using a Nd:YAG laser (1064 nm) for excitation, fluorescence and photo-induced sample damage are avoided. Further advantages of FT-RS in relation to conventional RS are the lower cost of equipment, the excellent spectral reproducibility and the ease of operation. On the other hand, since the Raman intensity is inversely proportional to the wavelength to the fourth power, FT-RS instruments

are intrinsically less sensitive than modern multichannel dispersive Raman spectrometers.

Fredericks and co-workers [126] used FT-RS to analyse aromatics on silica plates with and without an incorporated fluorescence indicator. Severe fluorescence was not observed in any case and the detection limit was ca. 10 μg . Plates with a polymer backing were found to be unsuitable because they clearly produced polymer bands in the Raman spectrum.

A feasibility study on in-situ TLC-FT-RS was performed by Everall et al. [127] using polymer additives as an example (Fig. 21). Of several common TLC adsorbents silica was found to be most appropriate. Significant background fluorescence was not seen, neither from the adsorbents nor from the adsorbates. For reliable identification, 100–200 μg of analyte has to be present. It has been shown that conventional visibly excited RS is, in principle, more sensitive but at the same time it often induces serious background fluorescence. Therefore, the authors believed that FT-RS is the more generally applicable technique.

Rau [128] analysed a mixture of paracetamol and salicylamide by TLC-FT-RS. A fluorescence free, identifiable spectrum of 40 μg paracetamol was observed. Comparing the TLC spectrum with reference Raman spectra, small shifts, broadening and change in relative intensity of

spectral bands were observed, which were due to the strong interaction of the adsorbate with silica.

Petty and Cahoon [129] also explored in-situ TLC-FT-RS analysing several pharmaceutical test compounds. About 10 μg of substance per TLC spot was necessary to obtain an identifiable spectrum. The use of FT-Raman spectral libraries for identification of the TLC spectra was discussed and different search algorithms were compared.

9.2. Resonance RS and surface enhanced RS

The major obstacle to the general appliance of RS is its moderate sensitivity. In certain cases, however, it is possible to significantly enhance the Raman signal by making use of resonance and/or surface effects. When the frequency of the exciting radiation coincides with an electronic absorption band of the analyte, so called resonance Raman (RR) spectra are obtained [130]. RR lines can be 10^2 – 10^6 times more intense than in normal RS. Armstrong and co-workers [131] applied RRS to characterise metalloporphyrins on silica gel plates. Using 514.5 nm excitation and an optical multichannel analyser, nanogram levels of the strong absorbing analytes could be measured. Increasing the laser power and focusing the laser spot did not improve the sensitivity, since it induced local heating and photodecomposition.

The Raman detection capability has been considerably enlarged by the discovery of surface enhanced Raman spectroscopy (SERS) in 1974 [132]. This is a technique in which lasers are used to excite vibrational transitions in molecules adsorbed on metallic substrates such as roughened silver electrodes or silver colloids [133,134]. As a result of large local optical field and resonance related effects, the Raman signal for the molecule on the metal surface is enhanced by factors up to 10^6 . This means that SERS can provide molecular recognition at the nanogram level or even lower. SERS detection in TLC can be accomplished by spraying the TLC spots with a silver hydrosol prior to the Raman measurement. The silver colloids are

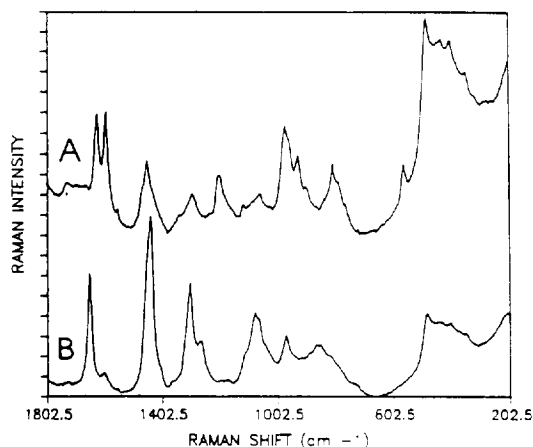


Fig. 21. TLC-FT-RS of (A) erucamide and (B) Irganox 1010 (200 μg each). From Ref. [127].

usually prepared by the reduction of silver nitrate with sodium borohydride [135] or sodium citrate [136]. Compared to normal Raman spectra, SER spectra often show small changes in relative intensity, band position and band shape.

Tran [137,138] was the first to report on the combination of planar chromatography and SERS. Three structurally similar dyes (85 ng each) were separated by paper chromatography and, after spraying with silver hydrosol, good-quality SER spectra were obtained in-situ using a conventional Raman spectrometer. The detailed spectra allowed identification and the limit of detection was 2 ng/cm².

During the last few years, the applicability and high-sensitivity of TLC–SERS has been demonstrated extensively by Koglin [89,139–143]. SER spectra of nucleic purine derivatives, benzoic acid and 1-nitropyrene spotted on silica plates, were obtained at the nanogram level [139,140]. Prior to the Raman measurement the TLC plates were sprayed to wetness with a borohydride silver sol and spectra were recorded using an Ar-ion laser (514.5 nm), a double monochromator and a cold photomultiplier. In two later studies [141,142], Koglin succeeded in dramatically improving the sensitivity of this methodology. In the first study a micro-Raman spectrometer consisting of a laser, a microscope, a triple monochromator and a multichannel detector, was used. With this system it was possible to acquire SER spectra of TLC spots of DNA bases (Fig. 22) and dibenzofuran at the low picogram level. Considering the fact that the laser focus is about 1 mm, the irradiated mass is only a few femtograms. By using a combination of SERS with RRS (SERRS) it was also possible to achieve picogram detection limits for TLC spots. Koglin [142] showed this for fluorescent compounds such as acridine orange (Fig. 23) and 2-aminofluorene, which have absorption bands at the used laser excitation wavelength (488.0 nm). It appeared that both the sample and background fluorescence, which normally swamp the Raman spectra, are almost totally quenched by the silver colloid particles. Koglin also demonstrated [143] that TLC–SERS could also be used to study the adsorption behaviour of cationic

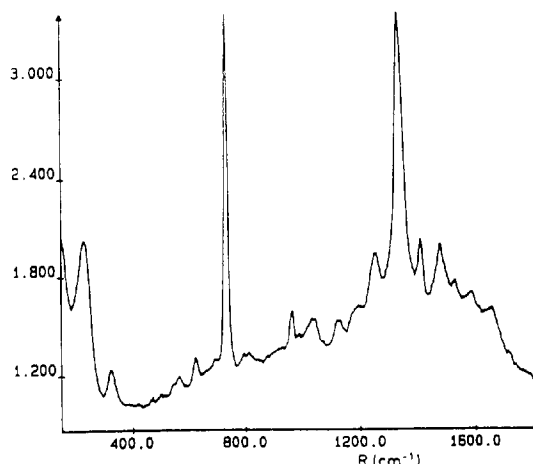


Fig. 22. Micro-SERS of 20 pg adenine spotted on silica TLC plate. Adapted from Ref. [141].

surfactant on silica gel. SERS was used (i) to assess adsorbate orientation and conformation, and (ii) to probe the effect of various environmental factors on interactions between the adsorbate and the silica–silver colloid substrate.

A thorough study on the influence of the TLC matrix in TLC–SERS has been performed by Soper and Kuwana [144]. The model compound pararosanine was mixed with silver sol prior to deposition on the TLC plate and analysed by a fiber optic Raman spectrometer. Various parameters such as the sol preparation protocol, the nature of TLC adsorbent and the time dependence of the SERS signal, were studied. Alumina was found to be the most appropriate TLC phase for sensitive detection of the pararosanine. Furthermore, it appeared that the TLC matrix stabilises the SERS signal by inhibiting the continued aggregation of the colloidal particles.

Recently, Rau [128] showed that it is possible to obtain TLC–SER spectra by excitation in the NIR region using an FT-Raman spectrometer. After spraying with a silver colloid solution, a good-quality FT-SER spectrum of a spot of 40 ng rhodamine B was recorded. Because of the NIR excitation, TLC plates containing fluorescent indicator could be used without any problem.

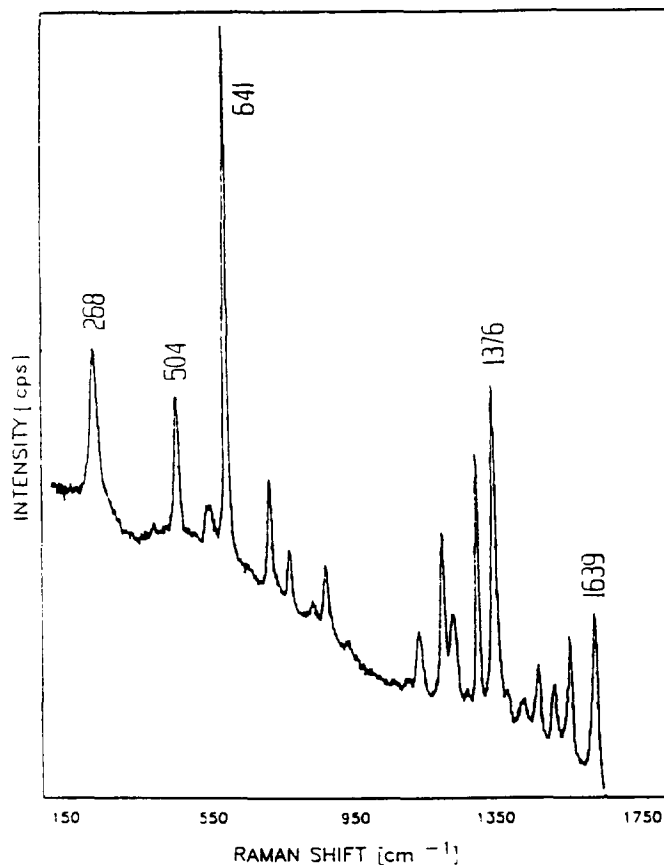


Fig. 23. SERRS of 30 pg acridine orange spotted on silica TLC plate. From Ref. [142].

Today, it is clear that SERS is a very interesting method for obtaining structural information about very small amounts of compounds separated by TLC. However, before any application is attempted, various factors should be considered. Prerequisite is the adsorption of the analytes to the silver hydrosol. Obviously, the character of both the analyte and the silver surface play an essential role here. Furthermore, size and dimensions of the silver colloid particles are very important and the production of stable, active and reproducible colloids is of major concern. The influence of experimental parameters such as the nature of the adsorbent, sol pH, sol activation, temperature, etc., may be strongly analyte dependent. In this respect, TLC-FTIR and TLC-RS are more generally applicable

methods, but it is evident that these techniques are considerably less sensitive than TLC-SERS.

10. TLC-fluorescence spectroscopy

Fluorescence spectrometry is used generally only as a quantitative detection tool in TLC. Compared with UV-Vis reflectance densitometry, fluorescence detection is much more sensitive, yielding detection limits in the low-pico-gram range. Baeyens and Lin Ling [145] reviewed the use of fluorescence densitometry for quantification in planar chromatography. In this section the use of fluorescence spectroscopy to identify TLC-separated compounds will be considered. First, the combination of TLC with

normal fluorescence excitation/emission spectroscopy (FEE) will be discussed and, secondly, the use of fluorescence line narrowing spectroscopy (FLN) will be treated.

10.1. Fluorescence excitation/emission

In comparison to absorption spectrometry, fluorescence spectrometry in general offers an enhanced selectivity because for each measurement two different wavelengths (excitation and emission) are involved. Furthermore, non-fluorescent UV-absorbing compounds are not detected. In principle, fluorescent substances can be characterised on the basis of their combined excitation and emission spectra. Most TLC scanning densitometers can be operated in the fluorescence mode and are able to record in-situ excitation spectra of TLC spots using a broad spectral source and a (motor-driven) monochromator. Densitometers, however, usually work under low-resolution conditions to maximize light throughput, and therefore the measured spectra are often not sufficiently characteristic for identification. Moreover, the emission envelope is usually determined by a cut-off or interference filter, so that continuous emission spectra can not be recorded.

Full excitation and emission spectra of TLC spots can be obtained using a conventional fluorescence spectrometer equipped with a plate scanning accessory. Such apparatus has, for instance, been used by Allen and Deutsch [146] to identify and quantify benzo[a]pyrene in cigarette smoke. As will be discussed in more detail later, Van de Nesse et al. [147] used the same spectroscopic set-up to confidently identify a number of PAHs in marine sediment, thereby illustrating the potential of TLC-FEE. Using in-situ fluorescence spectra for identification, one should realise that as result of the analyte-sorbent interactions, these spectra may show distinct differences with solution spectra. Also, a full, identifiable, spectrum requires a significantly greater amount of a substance than indicated by the fluorescence densitometric detection limit used for quantitative analysis.

Although not suitable for direct characterisa-

tion, common fluorescence scanning densitometers can be used to considerably improve the confidence of retention-based identification of fluorescent compounds. This has been shown by Poole and co-workers [148–150] who performed repeated scans of the same chromatogram at several fixed wavelengths, using the excitation monochromator and emission filters. Utilizing normalized emission response ratios determined at characteristic wavelengths, a procedure was developed to establish sample identity and to test for peak homogeneity. The TLC densitometric method was applied to the quantitative and qualitative determination of PAHs [149,150] in laboratory dust and crude diesel exhaust particulates. The technique was demonstrated to be accurate, reproducible and simple. Despite the sequential scanning at several wavelengths, this approach appeared to be less time-consuming than recording the full fluorescence spectra of each TLC spot.

Electronic scanning densitometers using vidicon tubes or charge-coupled device (CCD) cameras, have been developed mainly for TLC image analysis [151–157]. In principle such detectors can also be used to very rapidly acquire fluorescence emission spectra of TLC spots [158–160]. For this purpose a monochromator should be present between the plate and the multichannel detector. Thus, the spatial character of the detector is used for spectral analysis rather than for capturing the image of the entire TLC plate. Although suitable for fluorescence measurement, at present the commercially available TLC videoscanners cannot perform spectral analysis [161]. However, a few authors have reported some exploratory steps in this field.

Gianelli et al. [158] designed an optical system to obtain fluorescence spectra from each position along the elution axis of a unidimensional developed TLC plate, without need for mechanical scanning. With some porphines as test compounds, it was shown that overlapping spots could be resolved into their components using the obtained spectral data.

Cosgrove and Bilhorn [159] used a CCD camera to produce a TLC detector capable of two-dimensional mapping and quantification. By

introducing a monochromator between the plate and the camera, fluorescence emission spectra of individual compounds could be recorded, as was shown for a sample of anthracene.

Today the (potential) advantages of electronic scanning are clear. However, because of current equipment limitations image analysers cannot yet compete with conventional mechanical scanners [162]. Nevertheless, considering the on-going technological advances in this area, it is likely that in the future imaging analysis will be a viable detection method for TLC.

10.2. Fluorescence line narrowing spectroscopy

FLN is a laser-based high-resolution technique that requires cryogenic temperatures (<30 K) to provide vibrationally resolved fluorescence emission spectra. The FLN effect is induced by an energy selection in the excitation process. From the broad absorption band of the compound, only a small population of molecules, which exactly match the laser energy, is excited. The cold solid phase prevents reorientation of the excited molecules, so that the normally occurring inhomogeneous band broadening is avoided. The resulting emission spectrum is therefore vibrationally resolved and highly analyte-specific.

The applicability of FLN in (bio)analytical chemistry has been demonstrated extensively [163–166]. Usually the analytes are dissolved in glycerol–water which forms a transparent amorphous solid (glass) at low temperatures. Hofstraat and co-workers [167–169] have shown that FLN can also be applied to analytes adsorbed on to TLC plates. Highly-resolved spectra of pyrenes on silica plates could be obtained using an Ar-ion laser (363.8 nm) for excitation, a closed cycle helium refrigerator for sample cooling (down to 10 K) and a high-resolution monochromator to achieve instrumental bandwidths of 0.05–0.1 nm (Fig. 24). The FLN spectra permitted identification of structurally similar chloropyrenes. Quantitative application of TLC–FLN appeared to be feasible if an internal standard was employed and good linearity was observed in the low concentration range of the calibration plot. The detection limits were in the low nano-

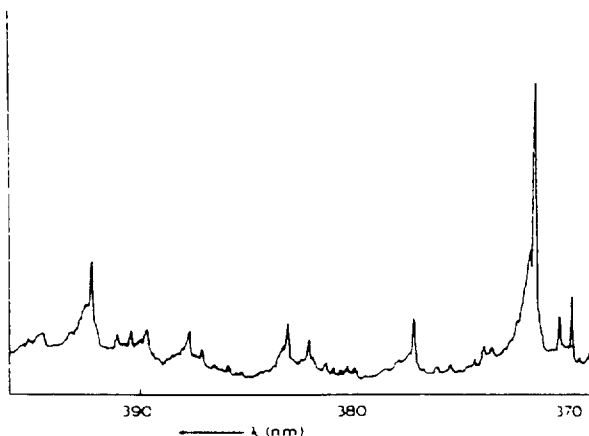


Fig. 24. FLN spectrum of pyrene (50 ng) on a silica TLC plate. From Ref. [168].

gram range. The authors stated that it should be possible to achieve a significantly better sensitivity using more sophisticated apparatus such as a tunable dye laser to excite at more favourable wavelengths, a multichannel detector and equipment for time resolved measurements to decrease laser stray light interferences. This has indeed been confirmed experimentally by later TLC–FLN studies (see also the section on coupled LC–TLC–fluorescence).

Cooper et al. [165,170] applied FLN to a PAH–nucleoside adduct adsorbed on a TLC plate. The experimental set-up included a pulsed dye laser and an intensified diode array detector yielding a detection limit of about 10 fmol (corresponding to ca. 4 pg adduct). A calibration curve over more than five decades of concentration was presented (in the absence of chromatography).

11. Coupling of LC with TLC and spectrometric detection techniques

In contrast with column liquid chromatography (LC), in TLC the sample is separated by distance rather than time. As discussed, when

the TLC development is terminated, the chromatogram is in fact immobilised and stored on the plate. The static TLC chromatogram can therefore allow examination of the sample zones over a long period of time. A variety of detection techniques can thus be applied, including time-consuming methods such as e.g. FTIR, FEE and FLN. These spectrometric methods are often not compatible with dynamic systems such as LC. It is possible, however, to link up the TLC detection potential with LC, via a coupling of the two techniques. On-line interfacing of LC and TLC can be accomplished by deposition of the effluent from the column on to the surface of a moving TLC plate, thereby immobilising (parts of) the LC chromatogram [171–177]. In an analogous fashion the combination of TLC with other separation techniques such as gas chromatography [178], supercritical fluid chromatography [179,180] and high-speed counter-current chromatography [181] can be achieved.

LC–TLC is clearly a multidimensional chromatographic technique and, when the LC column and TLC plate are of different selectivity, the combination can yield an enhanced separation efficiency. In this case, the deposited LC trace serves as the starting point for subsequent separation by means of TLC. When automated multiple development (AMD) is used as the second dimension, impressive separation capacities can be accomplished [171,173–175]. To maintain the resolution of the LC separation during interfacing and to prevent the TLC sorbent from lifting off the plate, it is necessary to minimize the amount of the LC eluent reaching the plate. Therefore, in LC–TLC the use of effluent-splitting or micro-bore LC in combination with a solvent elimination interface, is customary. Müller and Jork elegantly circumvented this limitation by interposing an on-line SPE step between the two chromatographic systems [177].

This section will focus on the use of LC–TLC for detection purposes. The applicability of FTIR, SERS, FEE and FLN for the off-line detection of LC chromatograms immobilised on TLC plates, will be discussed.

11.1. LC–TLC–FTIR

The on-line coupling of LC and FTIR using a flow cell is generally hampered by eluent interferences. Therefore, LC–FTIR is usually carried out by eliminating the solvent prior to IR detection of the solutes. This involves an interface which (partly) evaporates the eluent and deposits the analytes onto a substrate suitable for IR detection. Thus, the LC chromatogram is stored enabling efficient FTIR measurements, since both spectral resolution and signal-to-noise ratio strongly depend on the available analysis time.

Fujimoto and co-workers [182] used a TLC plate as deposition and IR-sampling medium after LC. The combination of the two separation modes was also shown to demonstrate increased chromatographic resolution. A mixture of four polymer additives could not be separated completely by micro size-exclusion chromatography alone. The SEC chromatogram was deposited continuously onto a moving silica plate, which was subsequently developed in a direction perpendicular to the direction of deposition thereby resolving all of the analytes. After development, in-situ DRIFT spectra were obtained of the individual spots, permitting identification of each additive. The limit of detection was reported to be about 1 μg .

In a second, similar study [183] micro reversed-phase LC was combined with alumina TLC to separate an eight-compound mixture. A DRIFT accessory (Analect) was used to allow recording of continuous IR chromatograms. The complete TLC plate could be imaged by FTIR and pure spectra of all individual compounds were obtained.

As described earlier, TLC sorbents are not the most ideal IR media, since they exhibit intense, interfering IR absorptions. These interferences can be circumvented using an IR-transparent deposition substrate instead of a TLC plate. Somsen et al. [184] used a solvent elimination device which was originally developed for LC–TLC, to deposit the effluent from a narrow-bore RPLC column onto a zinc selenide (ZnSe) sub-

strate. The deposited compounds were analysed by FTIR transmission microscopy. In this way, full and interference-free FTIR spectra could be obtained at a good sensitivity, yielding an identification limit of ca. 10 ng. The usefulness of the coupled system in the impurity profiling of pharmaceutical formulations was demonstrated [185]. Further, the potential of the LC–ZnSe–FTIR system to gain structural information about closely related compounds was illustrated by the analysis of a chlorinated pyrene sample [186]. A number of chloropyrene isomers and congeners present in the sample, which could not be characterised by MS, were separated by LC and unambiguously identified on the basis of the recorded FTIR spectra.

11.2. LC–TLC–SERS

Soper and co-workers [187] proposed that the LC–TLC concept be applied for SERS detection of LC separated compounds. The LC analytes were stored on the plate, so acquisition of the SER spectrum could be performed at any convenient time and long signal integration times can be used. A further advantage of the LC–TLC–SERS system in comparison to conventional solution phase SERS, is that the solid matrix prevents the continued aggregation of silver colloid–analyte complex, thereby giving stable signals for extended periods of time. Moreover, using alumina as TLC sorbent, enhanced SERS intensities were observed for the test compound parosaniline.

In these experiments the effluent from micro-bore LC column was deposited stepwise onto the TLC plate. Before Raman measurement, activated sol was added to the plate allowing the analyte to adsorb at the colloid silver surface. Optical fibers carried the laser excitation light (514.5 nm) to the plate and the scattered light to the spectrometer which recorded the Raman spectra using a scanning monochromator and a photomultiplier. Linear SERS signals were found for parosaniline in the range 10^{-7} – 10^{-5} M and the limit of detection was about 200 pg. Since the LC eluent is removed prior to the

addition of sol, the SERS analysis is independent of the LC mobile phase composition.

11.3. LC–TLC–fluorescence spectroscopy

Fluorescence based identification of related analytes in a mixture often requires a chromatographic separation of the components prior to detection. LC has an excellent separation power but sensitive on-line identification by fluorescence spectroscopy is hampered by the relatively short measurement time available in a flow system. By coupling LC to TLC, such a time constraint is absent and fluorescence methods that involve scanning (FEE) or cryogenic temperatures (FLN) can be applied.

Off-line fluorescence detection of TLC-stored LC effluents was accomplished by Strojek et al. [188]. The experimental set-up was similar to the one used for the LC–TLC–SERS experiments described in Ref. 187. In this case a He–Cd laser is used to induce fluorescence of naphthalene dialdehyde (NDA) derivatised amino acids. The integrity of the chromatographic peak shape was shown to be retained during the deposition process and a detection limit of about 10^{-8} M (10 fmol) achieved. The TLC substrate was able to prevent the fluorescence quenching of NDA-labelled lysine which is observed in solution. The authors recognized the possibility of using the LC–TLC concept to acquire excitation and emission spectra from the deposited analytes.

Hofstraat and co-workers [169,189–191] showed that with a spray-jet-assembly interface the effluent from a narrow-bore LC column could be deposited on to a TLC plate without serious loss of chromatographic resolution. Using some model compounds, the feasibility of applying FEE and FLN as off-line detection methods in LC, was demonstrated [190]. Van de Nesse et al. [147] used their LC–TLC system (Fig. 25) for the identification of PAHs present in a marine sediment sample. Applying isocratic LC it appeared not to be possible to separate all PAHs in the sample. Therefore, the LC chromatogram was deposited on a 30% acetylated cellulose plate, which was perpendicularly de-

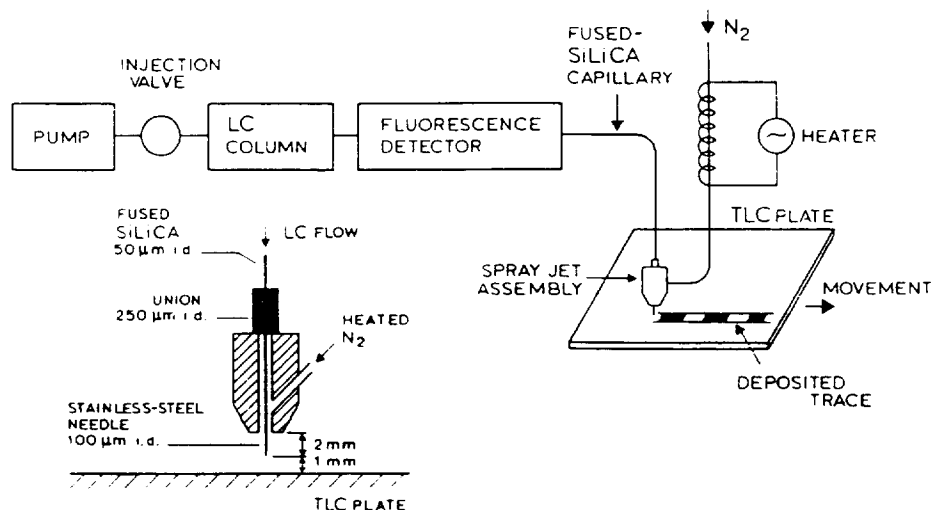


Fig. 25. Schematic of the spray-jet-assembly LC-TLC system. From Ref. [147].

veloped, thus performing two-dimensional chromatography. The overlapping peaks were then resolved completely (Fig. 26A). Using a fluorescence spectrometer with a TLC accessory, identification of the PAHs was accomplished on the basis of the recorded FEE spectra of the spots (Fig. 26B). The identification limits were in the low picogram range.

To distinguish between substituted PAHs originating from the same parent compound, the spectral selectivity of FEE is often insufficient. More information is provided by the vibrational structure hidden in the broad spectral bands which can be revealed by high-resolution techniques such as FLN. Because FLN requires extremely low temperatures, it obviously cannot be used as an on-line LC detection method. LC and FLN can however, be hyphenated through analyte storage on a TLC plate. The potential of LC-TLC-FLN has been demonstrated [192] by the analysis of an impure 1-chloropyrene sample. The RPLC chromatogram of the sample was deposited on a C_{18} modified silica plate and studied by FLN using a pulsed dye laser, an optical multichannel analyser and time-resolved detection principles. Pyrene and several mono-, di- and trichlorinated pyrenes present in the sam-

ple, were identified by the fingerprint structure of the vibrationally resolved spectra (Fig. 27). Although peak intensities varied, the spectral line positions appeared to be independent of the TLC matrix. The method allowed quantification (by standard addition) and compounds could be detected down to the low picogram level, which is about 100-fold better than in earlier TLC-FLN studies [168].

12. Conclusions

As shown by the examples provided in this review the combination of TLC with in-situ spectroscopy provides the analytical chemist with a powerful tool for the unambiguous identification of substances separated on their plates. The advantages of TLC in this type of work result in no small measure from the flexibility that the storage of the separation on the TLC plate provides. This enables the analyst to choose from a suite of post chromatographic characterisation techniques on the basis of the problem revealed by the separation. There is therefore no need to commit expensive and complex instrumentation to a problem which may not require

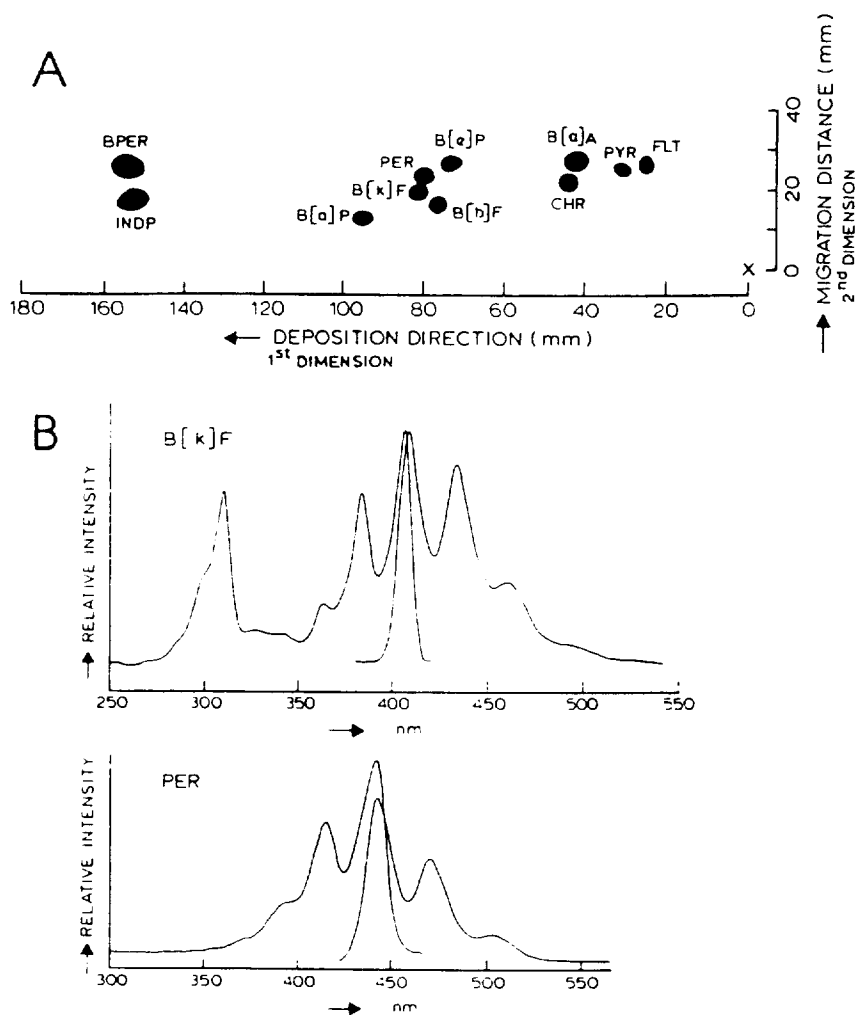


Fig. 26. (A) Two-dimensional LC-TLC of a marine sediment sample. First dimension: isocratic RP-LC; second dimension: 30% acetylated cellulose TLC. (B) In-situ FEE spectra of spot B[k]F and spot PER. Abbreviations: FLT = fluoranthene; PYR = pyrene; B[a]A = benz[a]anthracene; CHR = chrysene; B[e]P = benzo[e]pyrene; B[b]F = benzo[b]fluoranthene; PER = perylene; B[k]F = benzo[k]fluoranthene; B[a]P = benzo[a]pyrene; INDP = indeno[1,2,3-c,d]pyrene; BPER = benzo[ghi]perylene. Adapted from Ref. [147].

it, neither is there the need to repeat the chromatographic separation a second time to allow spectroscopy to be performed. In addition the plate can be physically transferred considerable distances from the site where the separation has been performed to the laboratory containing the spectrometer. A fuller appreciation of the advantages of TLC in combination with advanced spectroscopic characterisation, by both chro-

matographers and spectroscopists, should lead to the more widespread adoption of these techniques in both routine analysis and for problem solving. It might be expected that a similar appreciation of the potential of TLC by instrument manufacturers would result in the production of dedicated instrumentation which would further encourage the adoption of the approaches described here.

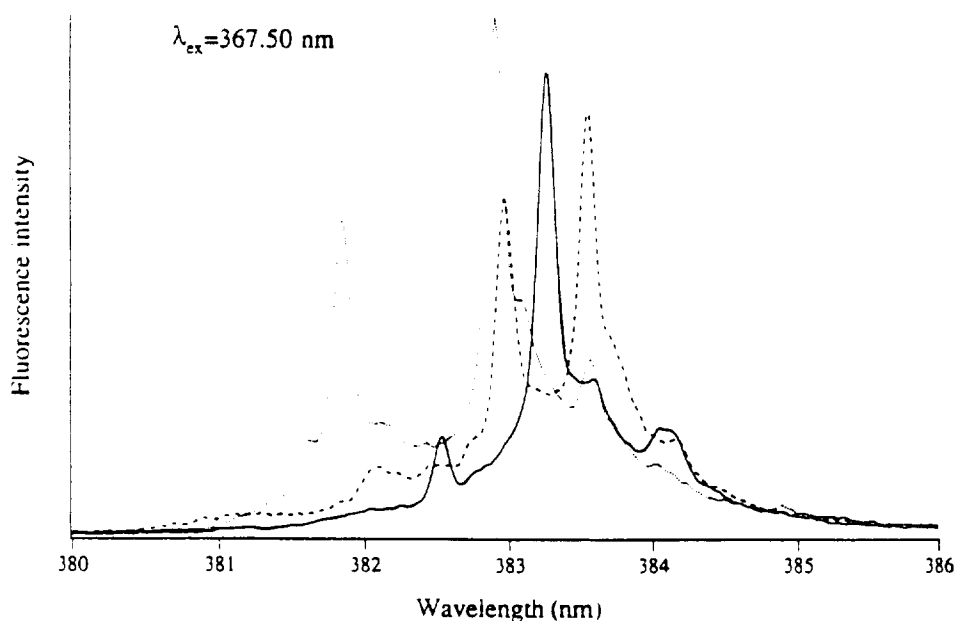


Fig. 27. In-situ FLN spectra of three dichloropyrene isomers recorded after TLC deposition of the LC chromatogram of an impure 1-chloropyrene sample. (---) 1,6-Dichloropyrene; (· · · ·) 1,8-dichloropyrene; (—) 1,3-dichloropyrene. Adapted from Ref. [192].

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