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REVIEW

# FLAT-BED CHROMATOGRAPHY ON IMPREGNATED LAYERS

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## 1. INTRODUCTION

Thin-layer chromatography (TLC), a flat-bed liquid chromatographic technique first introduced in the early 1950s by Kirchner and Stahl, appeared to be one of

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the most promising separation methods for samples that are not amenable to analysis by gas chromatography (GC). The number of papers involving TLC has increased rapidly, especially since improvements in several respects (plate technology, sample throughput, detection limit, separation efficiency and precision) led to high-performance TLC (HPTLC). This technique is widely employed both in industry and in research work[1–4] and compares well with high-performance column liquid chromatography (HPLC)[2]. The advantages of TLC are technical simplicity, economy, rapidity of analysis and the possibility of applying and developing simultaneously numerous samples and standards on the same plate under identical experimental conditions. Moreover, the chromatographic parameters can be easily adjusted and the success of the separation rapidly evaluated.

Further improvements in the selectivity of flat-bed chromatography and broadening of its applicability were realized by modifying the chromatographic properties of the supports (paper or thin layers) by chemical reactions or physical methods. Chemical modifications of the support are widely used in TLC; the groups of interest are chemically bonded to the reactive groups of the packing material of the layers. In the last decade many types of precoated alkylsilica bonded plates have become commercially available and papers reporting their use in reversed-phase (RP) TLC are increasing[5].

The simplest physical method is to adsorb on the supports chemically selective reagents with special reactive groups (*e.g.*, complexing or ion-pairing agents and ion exchangers) or liquids of very low volatility and very low or very high polarity (*e.g.*, paraffin oil or formamide, respectively). In the latter instance, the impregnating agents behave as stationary phases and by choosing the appropriate mobile phase, a partition separation process is obtained[2]. Acids, bases or buffers, which modify the pH of the layers, can be also used as impregnating agents. This impregnation technique, used earlier in paper chromatography (PC)[6], has been successfully tried in TLC and it is still widely applied in flat-bed chromatography on both paper and thin layers.

The aim of this review is to draw the attention of users of liquid chromatography to the possibilities of analysis offered by PC and TLC techniques on coated supports by surveying the wide variety of impregnating agents tested, the possibility of obtaining relationships between the chromatographic behaviour and chemical structure of a compound and the many practical applications. The review covers the subject from 1978 to 1987 and includes our own experience and comments. Some works published in 1988 are also mentioned. The literature prior to 1978 was covered in books by Stahl[7] and Kirchner[8], and the extensive general review on TLC and PC by Zweig and Sherma[1], Sherma and Fried[2,3] and Sherma[4] proved valuable in compiling this review. In the last 10 years several reviews dealing with certain aspects of TLC have appeared[9–12] and more specific ones will be cited where appropriate.

Studies in which chemically modified precoated plates or papers have been used, the first subject of a review by Brinkman[5], are not reported here.

Several impregnation techniques are used[8], depending on whether the plates are laboratory-made or precoated commercially. In the former instance, a solution of a coating agent is added to a slurry, whereas commercial plates are dipped, sprayed or chromatographed in a solution of the impregnating agent. A more uniform layer is generally obtained by the dipping technique. In order to obtain reproducible results, the preparation of impregnated plates or papers requires care and the method should

be standardized. In addition, in chromatographic experiments with impregnated layers, care should be taken that the eluent does not wash away or interact with the coating agent.

## 2. COMPLEXATION

The use of complexing agents plays an important role in liquid chromatography because it allows the separation of a large number of different analytes (organic compounds, metal ions, enantiomers) by interaction of two or more molecules or ions. An equilibrium process occurs and the formation of complexes changes the chromatographic behaviour of the compounds under examination.

### 2.1. *Charge transfer*

The need to separate a wide variety of aromatic compounds in environmental and toxicological analyses stimulated efforts to improve chromatographic techniques by incorporating electron acceptors or donors in the stationary phase. In this way a weak charge-transfer complex is formed by interaction between the analytes and the modified stationary phase. The complexation generally involves $\pi$-electrons, but $\sigma$- or $n$-orbitals and antibonding $\sigma$-orbitals can also play a role in the interactions.

In recent years, whereas silica materials chemically modified with electron donors or acceptors have been prepared and tested as packing materials for high-performance liquid chromatographic (HPLC) columns[13], impregnation techniques are still preferred in flat-bed chromatography. In the latter technique, silver salts and boric acid derivatives are the most frequently employed charge-transfer complexing agents.

2.1.1. *Silver salts.* Argentation chromatography, in which silver is used as a $\pi$-complexing metal, is widely used for the separation of organic compounds with electron-donor properties due to presence of unsaturated groups in the molecule. It has been hypothesized that the unsaturated group is attached to the metal by essentially a double bond, in which unsaturation electrons from the organic molecule form a $\sigma$-coordinate bond with the metal and the metal in turn donates a pair of $d$-electrons to the organic molecule through a $\pi$-bond. The stability of the $\pi$-complexes depends on the number, type, geometry and position of the double bonds in the molecule of the analytes.

Most studies using silver complexation employ TLC, because the instability of silver at elevated temperatures and problems with reproducibility and column lifetime impose severe limitations on GC and HPLC methods, respectively[14,15].

Silver nitrate as the impregnating agent and silica gel layers as supports are usually employed in argentation TLC. Recently several studies were made to test the effect of the anion of different silver salts on the $R_F$ values. The anion can influence the stability of silver–olefin complexes; thus silver sulphamate proved to be very suitable for the separation of fatty acid cholesteryl esters[16]; silver benzenesulphonate allowed the critical separation of *cis*-monoenoic esters and *trans,trans*-dienoic esters (*e.g. cis*-oleic and *trans,trans*-linoleic acids) which could not be achieved with silver nitrate[17]; silver iodate, which is a stronger complexing agent than silver nitrate, gave a better resolution of some terpenoids in some instances and in others it was at least as good as silver nitrate[18]. Ammoniacal silver-impregnated plates gave a better

separation of fatty acid methyl esters than did impregnated plates prepared from aqueous silver solution[19].

The influence of silver nitrate concentration[20,21] and of the coating material on the thin layer[21,22] have been studied. A 2.5% concentration of silver nitrate reduced the resolution whereas plates treated 5 and 10% silver nitrate behave identically[20]. Generally a 5% concentration has been preferred by many workers[18,20,23–25].

As regards the thin layer, Breuer *et al.*[22], employing silver-impregnated alumina plates, separated fatty acid methyl esters (FAMEs) with different carbon chain lengths and fatty acid mixtures with a significant improvement in the time required over that using silica gel impregnated layers, whereas Inomata *et al.*[21] found HPTLC silica gel plates to be unsuitable for argentation TLC because of rapid darkening during the activation process.

Silver-impregnated silica gel layers are employed to separate analytes differing in geometry (*cis/trans*-$\Delta^{11}$-octadecenol and *cis/trans*-$\Delta^{11}$-octadecenic acid[25]; *cis/trans*-fatty acid monoenoic esters and *cis/trans*-dienoic esters[17]), the position of the double bond, as in pentacyclic triterpene monools and diols[26] and different numbers of double bonds[20,22,23,25–28]. Generally, additional double bonds in the side-chain lead to a decrease in the $R_F$ values. The active adsorptive sites of the silica gel layers were found to interact also after silver impregnation[23]. In this study of the chromatographic behaviour of $\beta$-carotene, the $R_F$ values were attributed to the combined effects of the two different type of interactions. In addition, the polarity of the solvent systems influenced the retention mechanism: interactions with silver ions prevailed with less polar solvents whereas interactions with the residual silanol groups prevailed with more polar solvents.

Two-dimensional TLC was employed for the complete separation of analytes with different chain lengths and degree of unsaturation as labelled fatty acid methyl esters[29] and 5-*n*-alk(en)ylresorcinol homologues[27]. In particular, silica gel plates impregnated side-by-side with silver nitrate and paraffin oil or decane, forming coupled two-phase layers, were used. In this way different retention mechanisms dictated the chromatographic behaviour of the analytes: in one direction the separation of compounds with different degrees of unsaturation and in the other direction with different chain length is achieved on silver-impregnated silica gel and reversed-phase layers.

It is interesting that argentation chromatography may be used most effectively in conjunction with other chromatographic techniques. The fact that it affects separations according to the degree and type of unsaturation, with little if any separation of compounds with different chain lengths, makes it particularly suitable for combination with other partition methods (GC and HPLC). Breuer *et al.*[22] described a method of argentation TLC combined with GC for analysing complex mixtures of positional and geometric isomers of fatty acid methyl esters. Combination with HPLC has been used for the quantitative analysis of subclasses (alk-1-enylacyl, alkylacyl and diacyl types) and molecular species within each subclass of glycerophosphatides[30] and for the study of the metabolism of species of a given lysophospholipid class[24].

As regards analytical applications, in addition to those mentioned above, the separation of prostaglandins[31,32] and *p*-nitrobenzyl esters of giberellins[28], the isolation of *trans*-hexadecenoic and *trans*-octadecenoic fatty acid methyl esters from

lipid extracts[33] and the determination of triazine and chlorophenoxy acid herbicides in natural waters[34] and cholesterol in egg yolk[35] have been reported.

In conclusion, silver-impregnated TLC is now being successfully employed for analytical purposes and there are relatively few papers devoted to the study of parameters that can improve the separation process.

2.1.2. *Boric acid and borates.* Flat-bed chromatography using boric acid and its derivatives as impregnating agents for paper or silica gel layers is generally employed for the separation of polyhydroxyl compounds (carbohydrates, fatty acids, polyalcohols). Vicinal hydroxyl groups in a position and conformation favourable for the formation of a cyclic boric acid derivative and a polarity of the derivative different from that of the free diol are required of the analytes. The position of the active pair of hydroxyl groups influences the stability of the cyclic boric acid derivative and the retention of the analytes. As an example, the relative mobilities of cardenolides containing active hydroxyl groups only in the carbohydrate chain are reduced by boric acid, whereas the mobility of cardenolides with active hydroxyl groups in the genin part is considerably increased in the presence of boric acid[36]. This difference is probably related to the tetrahedral state of the boron atom in the cyclic sugar ester compared with a trigonal state in the genin ester.

Megges *et al.* studied the influence of phenylboronic and diphenylborinic acids[37] and of some borates[38] on the PC mobilities of cardenolides and bufadienolides and concluded that there are two requirements for the interaction of *cis*-1,2- or *cis*-1,3-diol groups with these acids: the capability of the diol to reach an O–O distance like that in phenylboronic acid esters or diphenylborinic acid complexes and the absence of considerable steric hindrance from a substituent near the reactive diol group.

Boric acid-impregnated plates have been used for the TLC resolution of various lipids[39–42], for the detection of lactulose in milk[43] and for the assay of ribonucleotide reductase[44].

2.1.3. *Other agents.* Several attempts have been made to employ other electron acceptors and donors in TLC with the formation of charge-transfer complexes. Weak electron donors may be small unsaturated or aromatic hydrocarbons with or without an electron-releasing substituent such as an alkyl, alkoxy or amino group. Weak electron acceptors may be aromatic or unsaturated compounds containing electron-withdrawing substituents such as $NO_2$, Cl or CN groups.

As a weak electron donor, Jain and Agarwal[45] employed *p*-toluidine as an impregnating agent of silica gel layers for the identification of trace amounts of 1-(2,4-dinitrophenyl)-3,5-dimethyl- or -diphenyl-4-arylazopyrazoles.

For the separation and identification of some hydroxyacetophenones, impregnation of silica gel with chlorobenzene rather than nitrobenzene was preferred[46]. Chlorobenzene impregnation also improved the separation of lichen acids of the pulvinic acid series compared with unimpregnated plates[47].

Slifkin and co-workers[48–50] studied the complexing effect of amino acids, nucleic acid bases and other organic acceptors on different analytes in charge-transfer TLC. They measured the interaction between the analytes and the impregnant by a binding constant $B$, defined as

$$B = \frac{R_F - R'_F}{R_F} \cdot 100$$

where $R_F$ is the value obtained in the absence and $R'_F$ that in the presence of the impregnant. In this way, they determined the effect of the structure and concentration of the impregnant on charge-transfer complex formation.

In conclusion the papers discussed reveal that in flat-bed chromatography over the last decade silver salts and boric acid are the most extensively employed charge-transfer impregnating agents. In contrast to HPLC, where silica and organic polymers modified with covalently bound electron donors or acceptors are still widely tested[13], relatively little work has been published on other possible charge-transfer complexing agents in TLC.

2.2. *Coordination bonds*

The formation of complexes between a metal ion and an electron-donor molecule with at least one free pair of electrons can be employed for the separation of organic compounds or metal ions. The thin layer or the paper is impregnated with the appropriate metal salts[51–70] or with the ligands[71–81], respectively. In both instances the resolution of a complex mixture is correlated with the complex stability constant, which in turn depends on the structure of the compounds and on the polarity of the solvent.

2.2.1. *Metal ions as impregnating agents.* The impregnation of adsorbents with transition metal salts has been of great utility in the separation of different classes of compounds: alkaloids[51], sulpha drugs[52], carbamates[53], phenolic acids[54–56], amino acids[57–59], PTH-amino acids[60–62], dyes[63–65], barbiturates[66], carbohydrates[67,68] and organic acids[69].

Most of the reported systems improved the resolution of analytes[51–69] and spots with reduced tailing[51–53,60,64,66] compared with those on bare thin-layer plates.

In TLC, the complexation of phenolic acids or aldehydes[54–56] with impregnated $Fe^{III}$ ions, depending on the eluent system, caused a decrease in retention of the analytes respect to that on plain silica gel plates, where hydrogen bonding is the predominant interaction.

Bhushan and co-workers[57–62] studied the resolution of amino acids and their PTH derivatives on silica gel plates impregnated with different transition metal ions at various concentrations in different eluent systems. In all instances the time required for developing the impregnated layers is less than that for plain silica gel. $Zn^{II}$-impregnated silica gel plates showed a greater ability to form complexes with amino acids[57] and their PTH derivatives[60] than $Cd^{II}$- and $Hg^{II}$-impregnated plates, whereas $Fe^{II}$ and $Ni^{II}$ were the best impregnants for the resolution of PTH-amino acids[61] when compared with transition metals of the same period ($Co^{II}$, $Mn^{II}$, $Cr^{III}$, $Cu^{II}$, $Zn^{II}$). Alkaline earth metal-impregnated silica gel plates showed a similar chromatographic behaviour to bare silica gel with respect to amino acids[59].

The $R_F$ values were also influenced by the nature of anions present in metal salt[52,53,63,65,66]; acetate anion generally gave the best results[52,53,63,65].

A rapid method of resolving mixtures of some commonly occurring carbohydrates on cellulose or paper impregnated with tungstate was reported by Briggs and co-workers[67,68], with comparable results on the two supports.

2.2.2. *Ligands as impregnating agents.* The TLC separation of different metal ions has been attempted by many workers by impregnating the layer with a complexing agent[70–87].

Various compounds with amino[70–73] or acid[74–77] groups, salts of penicillin G (PG) or penicillin V (PV)[78], 8-hydroxyquinoline[79], dibenzoylmethane[79] and diantipyrylmethane solutions containing potassium iodide or ammonium thiocyanate[80] are considered good complexing agents and capable of separating different metal ions. In addition, a sparingly soluble compound such as a Schiff base, forming a complex with copper(II) even less soluble than the impregnant itself, permitted the determination of microgram amounts of the test ions on impregnated paper[81].

Other complexing agents such as digitonin and EDTA have been employed for the separation of sterols[82] and tetracyclines[83–87], respectively. 3$\beta$-Hydroxysterols with or without a C-22 double bond were fractionated in accordance with the length of the side-chain; the analytes with longer side-chains complex faster than digitonin and move more slowly on the TLC plates[82]. Generally, tetracyclines show tailing on the bare silica gel layers because of their chelation with trace metals present as impurities in the silica gel. In order to avoid this, HPTLC plates are generally impregnated with $Na_2EDTA$ and then activated. In this way, simple, rapid and sensitive methods for the separation of tetracyclines from their major degradation products were developed[83–86]. The separation and semiquantitative determination of tetracycline degradation products in tetracycline hydrochloride powders and capsules on cellulose impregnated with EDTA was reported[87].

### 2.3. *Ligand exchange*

Ligand-exchange chromatography (LEC) is a technique mainly employed for the separation of compounds according to their ability to enter inside the coordination sphere of the complex-forming ion. This mode differs from ion-exchange, adsorption and other types of chromatography in its basic process of interaction between the analyte and stationary phase. The functional groups of the packing material (ion-exchange resin, silica gel) enter into the coordination sphere of the metals acting as fixed ligands. In this way an analyte (A), acting as a mobile ligand, coordinates the stationary complex (RM) by an equilibrium reaction:

$$RM + A \rightleftharpoons RMA$$

The selectivity and resolution of the chromatographic process are related to the different stabilities of the complex species (RMA) according to the physical and/or chemical properties of the analytes.

LEC offers interesting possibilities for the separation of various classes of compounds[88–92]. Various amines were separated qualitatively on impregnated papers and quantitatively on columns of $Cu^{II}$-impregnated zinc silicate by Singh and Darbari[88]. Mixtures of carbohydrates were separated according to their complex stabilities by TLC employing copper ion and a mixture of silica gel and Ionex 25-SA[89]. For comparison purpose complexes with other metal ions were investigated but the extent of complex formation was greatest with copper(II) ions. A successfully application of LEC was reported by Antonelli *et al.*[92] for the analysis of a mixture of $\alpha$-amino acids, their corresponding $\beta$-isomers and some dipeptides. $Ni^{II}$ and $Cu^{II}$ in the form of $M(NH_3)_x$ were the metal ions chosen because both formed sufficiently strong and appreciably different complex species with the considered ligands. The best exchanger was $Ni(NH_3)_6^{2+}$, which was able to separate the analytes according to the

stability of the corresponding coordination compounds in aqueous solution. Data obtained by TLC were advantageously translated to column chromatography and comparable results were obtained[90]. The separation and determination of sulphur compounds in high-boiling petroleum distillates was obtained on mercury(II) acetate-impregnated silica gel plates with *n*-hexane as developing solvent[91].

An important application of LEC is the separation of optically active substances without any previous conversion into diastereomers. In the last 10 years, many workers have used chiral stationary phases instead of chiral mobile phase additives for the direct separation of enantiomers by TLC and HPLC with reversed-phase systems. Davankov and Semechkin[93], Armstrong[94], Mehta[95] and Günther[96] have discussed in detail these chiral separations by liquid chromatography based on the formation of an enantioselective ternary complex formed by a chiral reagent of a definite steric configuraton (fixed ligand) adsorbed on the stationary phase (generally $C_{18}$ bonded silica gel), a metal ion (*e.g.*, $Cu^{II}$) or one or another of the two enantiomeric analytes (mobile ligand). Therefore, the two mixed-ligand diastereomeric complexes formed possess different stabilities if the fixed ligand (resolving selector) recognizes the configuration of the mobile ligand. The chiral reagent employed was generally an amino acid with a pronounced hydrophilic character that displays the largest enantioselective recognition ability with $Cu^{II}$ complexes. In order to obtain strong adsorption of the chiral selector on the stationary phase, a reversed-phase surface and a aqueous–organic eluent were employed.

A suitable chiral selector was (2*S*,4*R*,2′*RS*)-4-hydroxy-1-(2′-hydroxydodecyl)-proline. As regards chiral TLC investigations, Günther *et al.* [97] obtained chiral layers by treating an octadecyl-modified silica TLC plate with a solution of copper(II) acetate followed by a solution of the above chiral selector; a simple and rapid method of monitoring optical purity was obtained. These plates, now commercially available, allowed the separation of optical isomers of amino acids, N-methylamino acids, N-formylamino acids, dipeptides and N-carbamyltryptophan[98–103].

Another chiral selector employed for the separation of Dns-amino acids was N,N-di-*n*-propyl-L-alanine (DPA)[104,105]. Grinberg and Weinstein[105] introduced a two-dimensional RP-TLC technique in order to separate a large number of Dns-amino acids more effectively than in a previous study[104]. In the first direction, the separation of Dns-amino acids in a non-chiral mode was achieved with an elution gradient (aqueous sodium acetate buffer–acetonitrile). A temperature gradient was applied to the plates treated with the chiral copper(II) complexes of DPA during the development in the second dimension, and an enantiomeric separation was obtained. Another application of the two-dimensional separation of D,L-Dns-amino acids was described by Marchelli *et al.*[106]. They prepared different chiral ligands in which two L-amino acids were joined via an amide bond by ethylene (AA-NN-2) or trimethylene bridges (AA-NN-3). Small variations in the length of the *n*-alkyl chain and in the lipophilicity of the amino acid strongly influenced the stereoselectivity: ligands of the AA-NN-3 type gave poor or no resolution whereas those of the AA-NN-2 type gave satisfactory results. The best separation was achieved with the copper complex of Phe-NN-2.

A new chiral selector (poly-L-phenylalaninamide) was prepared easily and without tedious purification procedures by Sinibaldi *et al.*[107]. Poly-L-phenylalanin-amide–$Cu^{II}$ complex-impregnated $RP_{18}$ silica gel plates separated a large number of

Dns-amino acid enantiomers using water–acetonitrile mixtures as the eluent under isocratic conditions.

The studies reported above indicated that several parameters influence the retention and enantioselectivity of the chromatographic system: (i) the $Cu^{II}$ concentration necessary for the impregnation of the plate —the best concentration for the separation of D,L-Dns-amino acids is 3 or 4 m$M$[103–105,107]; (ii) the pH of the eluent— solutions are generally employed in the pH range 5–8 because the amino acids must act as bidentate ligands and therefore it is necessary to release the proton from the ammonium group[103–105,108]; (iii) temperature —a decrease in temperature generally results in improved separations[103,105]; and (iv) the water to organic modifier ratio in the eluent— the experimental conditions depend on the polarity of the analytes.

In addition, it is noteworthy that the L-enantiomer is generally more strongly retained than the D-enantiomer[97,103–105,107], depending on stereochemical interactions between the chiral selector and the analyte.

In conclusion, LEC could contribute to the solution of a number of problems, in particular the separation of optical isomers, which is of great interest in the design of novel pharmaceuticals, where isomers may have different effects on an organism; one isomer may be inactive or even harmful.

### 2.4. *Inclusion complexes*

The torus-shaped cyclic oligosaccharides α- and β-cyclodextrins (α- and β-CD), made up of six or seven α-1,4-linked D-glucopyranose units, respectively, can selectively include in their central cavity various inorganic and organic molecules and ions[109]. The fit of guest molecules in the CD (host) cavity determines the stability of the inclusion compounds and the selectivity of the complexation process. This property of CDs has been used to advantage in liquid chromatography in the last 10 years; in HPLC and in TLC the separation of various compounds and isomers through CD complexation was achieved using CDs as mobile phase components in RP systems[110,111]. Recently, chemically bonded CD–silica stationary phases have become commercially available as packing materials for HPLC columns[112].

Comparative studies on the application of α- and β-CD as mobile phase components for the separation of various isomers by RP-TLC and RP-HPLC on $C_{18}$ layers and columns were recently reported[113]. In TLC experiments, before the usual chromatographic development, it was necessary to pretreat the $C_{18}$ plates with the CD solution to be used as mobile phase. With both techniques the separation of *ortho*-, *meta*- and *para*-substituted benzenes was obtained. With 1- and 2-substituted naphthalenes only one peak was eluted (2-substituted naphthalenes) because isomers substituted in position 1 were irreversibly adsorbed on the RP-18 column. In such a case, only TLC can be used for evaluating the composition of mixtures of this type of isomer.

Studies on the stability of various CD complexes were also performed by using RP-TLC[105,114–120]. In these experiments, in order to obtain a reversed-phase chromatograpic system, the layers were coated with paraffin oil. Several hydrophilic β-CD derivatives are widely utilized for the stabilization and solubilization of drugs, pesticides, etc. [109]. The cyclodextrins are generally more hydrophilic than the guest compound and as a consequence the CD complexes should be more hydrophilic than the original guest compound. This lipophilicity difference could be measured by

RP-TLC by determining the variation in the $R_F$ value of a compound in a chromatographic system in the presence and absence of β-CD soluble derivatives[114]. The larger the difference in $R_F$ values, the higher is the stability of the complex. Changes in the guest molecule structure allow structure–complex stability correlations to be studied.

Cserháti and co-workers employed RP-TLC to study inclusion complexes formed by a water-soluble β-CD polymer (SCDP) and various compounds such as polymixyn (an antibiotic)[115], *sym*-triazine[116], triphenylmethane[117], nitrostyrene[118] and barbituric acid derivatives[119] and chlorophenols[108]. Paraffin oil-coated silica gel plates were employed as the stationary phase and ethanol– or methanol–water solutions of SCDP as the eluent. Experiments with the guest molecules dissolved in the eluent were also performed[120]. The effect on the stability of the complexes of the percentage of organic modifier in the eluent, salts differing in their charge and the radii of the cations and basic and acidic environments were investigated. An increasing proportion of organic modifier or an increasing concentration of the salts and/or cations with greater ionic radii reduced the stability of the SCDP–polymixyn complex[94]. With barbituric acid derivatives, the stability of the complex increased with branching or increasing chain length of the alkyl substituents[119]. Basic and acidic environments decreased[108] whereas sodium chloride had little effect on the stability of the complex[108,120]. The formation of an inclusion complex between SCDP and chlorophenols was confirmed by $^1$H NMR spectroscopy.

TLC was also employed by Schneider *et al.*[121] to examine host–guest interactions between another macrocyclic molecule, an azacyclophane derivative immobilized on silica gel layers, and numerous azo dyes as guest molecules. As a result, only compounds with acidic groups, particularly $SO_3H$, were selectively bonded. According to the selectivity found in TLC experiments and spectroscopic investigations in solution, the essential factors in complexation are the lipophilic–hydrophobic binding in the cavity of the macrocycle and the electrostatic attraction between polar substituents in the substrate and the positive charges in the host molecule.

From the results obtained, it seems that RP-TLC on impregnated plates, a technique that has the advantages of being very easy to carry out and not requiring complicated instrumentation, is suitable for characterizing the stability of inclusion complexes.

## 3. ION EXCHANGE

Insoluble salts of polybasic acids with tetravalent metals (phosphates, arsenates, molybdate and tungstate of zirconium, titanium and cerium), ammonium salts of heteropoly acids (molybdophosphate, molybdoarsenate, molybdosilicate and tungstophosphate) and hydrous oxides have been extensively investigated as ion-exchange materials in column and flat-bed chromatography[122,123]. Zirconium phosphate-impregnated papers have been used for separations of inorganic ions[124], amino acids[125] and alkaloids[126].

The advantages of chromatography on papers coated with inorganic ion exchangers over partition chromatography on plain paper are the possibility of choosing an exchanger with a marked selectivity for a given compound and the use of aqueous solutions of inorganic salts as eluents. In selecting other ion-exchange systems for the separation of inorganic ions, Testa[127] showed that papers impregnated with

liquid ion exchangers, such as tri-*n*-octylamine (TOA), were good separation media. The suitability of these ion-exchange paper systems for the separation of a wide variety of compounds has been reviewed by Brinkman[128].

In recent years, as expected considering the decreasing interest in inorganic ion exchangers as column packing materials, few workers[129–139] have utilized this type of ion-exchange chromatography on paper or thin layers for separations of inorganic ions[129–132] or organic compounds[133–139]. In most of the studies[129–136,138,139], the effect of the ion exchanger was evaluated by comparing retentions on plain paper (or thin layer) and on the corresponding coated systems. For the separation of inorganic cations, aqueous acid–organic modifier mixtures [dimethyl sulphoxide (DMSO)[129], *n*-propanol[130,131] or benzene[132]] were tested as eluents. On tin(IV) arsenate paper, DMSO–aqueous nitric acid was found to be almost specific for $In^{III}$ by Qureshi and Sharma[129], and the separation of $In^{III}$ from $Ga^{III}$ and $Al^{III}$ was reported. Two-dimensional TLC on silica gel–pyridinium tungstate layers allowed the separation of numerous amino acids; a linear relationship between the $R_M$ values of several amino acids and the number of carbon atoms was demonstrated by Srivastava *et al.*[134]. Separations of alkaloid mixtures containing up to five compounds were achieved within 15 min on zirconium antimonate paper[135], and zinc silicate papers were used for the separation of phenols[138] or amines[139] by Rawat and co-workers. In addition, papers impregnated with liquid anion exchangers have been used by several workers[140–144]. Tri-*n*-octylamine salts and Aliquat 336 were generally tested and the separation of anions by using mixtures of these liquid anion exchangers with aqueous inorganic[140] or organic[141] acids was reported. A comparison of the chromatographic behaviours of 25 steroidal glucosiduronic esters on normal- and reversed-phase systems was reported by Mattox and Litwiller[144]. Using the same liquid anion exchanger, the resolving properties of the two systems were markedly different, increasing the possibility of separation of a given mixture.

With synthetic insoluble inorganic exchangers, the ion-exchange papers are usually prepared by precipitation of the desired impregnating agents in the paper[124] and with liquid anion exchangers by dipping the papers in a solution of the exchanger in an organic solvent[127]. In earlier TLC experiments with ion exchangers, the material on the plates was simply the exchanger mixed with starch or plaster of Paris as binder[8]. More recently, better results and stable thin layers were obtained by coating the plates with a mixture of silica gel and the inorganic material[132–134]. For TLC experiments, a slurry of the exchanger and silica gel (1:50 or 1:100, w/w) in the appropriate solvent is sprayed on the glass plates and dried under different conditions[132–134].

In conclusion, on inorganic exchange papers, the frequently noted elongation of the spots allowed the separation of only two compounds in many instances. By using liquid anion exchangers as impregnating agents, PC results can be useful for predicting the anion-exchange behaviour of anions in liquid–liquid extraction processes[141].

## 4. ION PAIRING

Over the years, a variety of different approaches have been adopted in order to achieve for ionic or ionizable species, such as compounds of biological or pharmaceutical interest, the resolution, sensitivity and rapidity expected of new HPLC systems, as these properties were difficult to obtain with the traditional ion-exchange

packing materials. In HPLC on polar or non-polar columns, increases in retention, reduction in tailing and improvements in selectivity have been achieved by adding to the eluent counterions of the opposite charge with respect to the samples and generally containing hydrocarbon chains[145–148]. For the separation of anions quaternary alkylammonium ions were employed, whereas for the separation of cations hydrophobic (alkylsulphates or -sulphonates) or hydrophilic ion-pairing (IP) reagents, such as inorganic anions, were used. Ion-pair formation between the analyte ions and the oppositely charged counter ions is assumed to be responsible for the observed increase in retention. Obviously, an appropriate pH can be selected in order to ensure that the analytes and the ion-pairing agents are in a charged form. Many terms have been proposed for this chromatographic technique, *e.g.*, ion-pair chromatography[145], soap chromatography[146], solvent-generated (dynamic) ion-exchange chromatography[147], hetaeric chromatography[148] and chromatography on sorbed ionic sites[149], reflecting the uncertainty which exists regarding the retention mechanism. Detailed discussions on the various proposed models can be found in reviews[150–152].

In contrast to column chromatography (HPLC), there have been relatively few applications of IP techniques in flat-bed chromatography, after a report appeared in the mid-1960s on the usefulness of paper impregnated with surface-active agents for the separation of dyes[153]. In TLC the first investigations were carried out by Lepri and co-workers[154–169] using alkyl-bonded silica gel layers and anionic or cationic surface-active agents as pairing ions. The necessity to impregnate the plates with the IP reagent before TLC was demonstrated, because by dissolving the surfactant only in the eluent, as in paper[153] or column chromatography[146], no change in the chromatographic behaviour of the analytes was observed[154]. Precoating of the plates before use has been confirmed to be necessary if IP reagents with long-chain alkyl groups are employed[170–175]. Indeed, if they were dissolved only in the eluent, it appeared to "demix", all of the spots ran with or very near to the solvent front and no formation of ion pairs was observed. In some instances, two spots appeared[175]. The lack of the effect observed was related to the lower mobility of the IP reagent with respect to the solvent on the layer material, which caused the analytes to be eluted before the counter ions reached them. By using short-alkyl-chain IP reagents, precoating of the plates was generally believed to be more efficient for ion-pair formation, especially for data transfer from TLC to HPLC[170,175]. However, some results with IP reagents of the second type, dissolved only in the eluent, have been reported[176–178].

Table 1 lists the IP reagents, the type of thin layer, the eluents and the analytes or analyte classes used in IP-TLC. The papers cited are ordered on the basis of the chemical nature of the IP reagents, *viz.*, surface-active, alkyl sulphates and sulphonates, quaternary alkylammonium and inorganic anions. Most of the work reported in Table 1 was aimed mainly at improving the separation characteristics of chromatographic systems and at showing the applicability of IP-TLC as a versatile method for the separation of a wide range of compounds[154–169, 171–173,185]. In many of them, comparisons between IP-TLC and other TLC systems were reported[156,158,160,161,164,165,168,169,173,185]. In several instances, IP-TLC was utilized as a rapid and efficient method for data transfer to HPLC[170,174,181] or for correlating the chromatographic behaviour of organic species with their chemical characteristics[180].

TABLE 1
ION-PAIR CHROMATOGRAPHY ON IMPREGNATED LAYERS

| *Impregnating agent* | *Thin-layer*[a] | *Eluents* | *Compounds or compound class* | *Ref.* |
|---|---|---|---|---|
| *(a) Surface active:* | | | | |
| *Anionic:* | | | | |
| (Triethanolamine) dodecylbenzensulphonate (DBS) | Silanized silica gel 60H (L) | Methanol–water acetic acid at different pH | Primary aliphatic and aromatic amines | 154,155,158 |
| | | (a) Methanol–water–acetic acid; (b) methanol–water | Sulphonamides | 157 |
| | | (a) Methanol–aqueous buffers (pH 5.0–11.3); (b) ammonia solutions in 20% methanol | Phenols | 161 |
| Dodecylbenzenesulphonic acid (H-DBS) | | Methanol–water–acetic acid at different pH (0.7–6.1) | Amino acids | 159 |
| | | (a) Methanol–water–acetic acid at different pH (1.2–8.1) | Peptides and dipeptides | 160,162 |
| | Silica RP-2; RP-84; RP-18 (C) | Methanol–water–acetic acid at different pH | Amino acids; dipeptides | 163 |
| | Silica RP-18 (C) | Methanol–water–acetic acid at different pH and ionic strength | Di- and polypeptides; amino sugars | 165 |
| | Silica RP-18; SIL $C_{18}$-50; $OPTI_{12}$-UPC (C) | Methanol–aqueous buffers or ammonia at different pH | Chloro-, bromo- and alkyl phenols | 166 |
| | Silica RP-18; SIL $C_{18}$-50 (C) | Methanol–water–acetic acid at different pH and ionic strength | Primary aliphatic mono- and diamines; amino acids | 165 |
| | Silica SIL $C_{18}$-50 (C) | Methanol–water–acetic acid at different pH | Diastereomeric di- and tripeptides | 168 |
| | Silica SIL $C_{18}$-50 (C) | Methanol–water–acetic acid | Amino acids and derivatives | 169 |
| | Silica $KC_{18}$ (C) | 1 *M* acetic acid + 0.2 *M* HCl in methanol–water (7:3) | Amino acids | 173 |
| Sodium dodecylsulphate (SDS) | Silica $KC_{18}F_{254}$ (C) | Aqueous solution of NaCl (3%) and SDS (0.2%)–acetonitrile–methanol (50:10:10), apparent pH 2.5 | Peptides | 172 |
| | Silica Si 60 $F_{254}$ (C) | 0.05 *M* ion-pairing agent in methanol–water (6:4) | Carboxylic and sulphonic acids; aliphatic and aromatic amines | 177 |
| *Cationic:* | | | | |
| N-Dodecylpyridinium-chloride (N-DPC) | Silanized silica gel 60 HF (C) | (a) Methanol–water–acetic acid; (b) methanol–water–acetic and hydrochloric acid | Water-soluble food dyes | 156 |

*(Continued on p. 14)*

TABLE 1 *(continued)*

| *Impregnating agent* | *Thin-layer*[a] | *Eluents* | *Compounds or compound class* | *Ref.* |
|---|---|---|---|---|
| | | Methanol–water at different pH (1.4–9.2) and ionic strength | Primary aromatic amines | 157 |
| | Silanized silica gel 60 HF (L); Silica RP-2 (C) | (a) Methanol–water–acetic acid; (b) methanol–water; (c) water | Polypeptides (MW 897–3495) | 164 |
| | Silica Sil $C_{18}$-50 (C) | 0.5 *M* sodium acetate in water–methanol (20%) | Diastereomeric di- and tripeptides | 168 |
| | Silica Sil $C_{18}$-50 (C) | (a) Methanol–aqueous ammonia buffer at different concentration; (b) hexane–ethyl acetate–acetic acid (67:32:1) | Indole derivatives | 167 |
| *(b) Alkylsulphonic acids and -sulphonates:* | | | | |
| Heptanesulphonic acid (HSA) | HPTLC silica RP-2; RP-8; RP-18 (C) | 0.25–3% HSA in methanol–water–glacial acetic acid (80:15:5) | Phenothiazine bases and sulphoxides | 176 |
| | RP-8 $F_{254}$ (N-15388); RP-18 $F_{254}$ (N-15389) (usable at any water content) | 0.2% HSA in methanol–water–glacial acetic acid in different proportions | Alkaloids | 179 |
| Sodium heptylmethyl-sulphonate | Silica Si $F_{254}$ (C) | 0.05 *M* ion pairing agent in methanol–water (6:4) | Aromatic amines | 180 |
| *(c) Quaternary alkylammonium salts:* | | | | |
| Cetyltrimethylammonium bromide (CTMA) | Silica gel (C) | 0.1 *M* CTMA in methanol–acetone (9:1) with or without 1% glacial acetic acid | Ionic food dyes (sulphonates) | 171 |
| Tetradecyltrimethyl-ammonium bromide (cetrimide, CTA) | Silica Sil G/$UV_{254}$; Silica 60 $F_{254}$; Silica RP-$KC_{18}$ (C) | 0.1 *M* CTA in phosphate buffer–methanol (or aceto-nitrile or tetrahydrofuran) (pH 5.0) | Carboxylic and sulphonic acids; phenols | 174,181 |
| Cetrimide; tetrabutyl (or hexyl, octyl, dodecyl)ammonium bromide | HPTLC silica RP-2; RP-8; RP-18 (C) | Methanol–water + 0.1–0.5% ion-pairing agent | Hydrophilic food dyes | 170 |
| Tetrabutyl (or methyl)ammonium halides | Silica Si $F_{254}$ (C) | 0.05 *M* ion-pairing agent in methanol–water (6:4) | Carboxylic and sulphonic acids | 180 |
| Tetramethyl (or propyl, *n*-butyl, heptyl)ammonium halide (TMA, TPA, TBA, THA) Cetrimide; Cetyl (or dodecyl)trimethyl-ammonium bromide (CTA, DTA) | Silica gel; paraffin-coated silica gel; silica RP-$C_{18}$ (C) | Methanol–phosphate buffer (pH 2–11) | Hydroxybenzoic acids; aspirin and anti-pyrene metabolites | 175,182 |

TABLE 1 *(continued)*

| *Impregnating agent* | *Thin-layer*[a] | *Eluents* | *Compounds or compound class* | *Ref.* |
|---|---|---|---|---|
| Hexyl (or octyl, decyl)trimethylammonium halide; cetrimide | Paraffin-coated silica gel (C) | Methanol–water at different pH | 2,5- (or 2,6-)Dihydroxybenzoic acid; gentisic and salicylic acids | 183 |
| 1,3-Bis(trimethylammonium)propyl bromide | Silica gel; paraffin-coated silica gel; silica RP-$C_{18}$ (C) | Methanol–water; methanol–dichloromethane | 2,4-(or 2,6-) dihydroxybenzoic acid; gentisic and salicylic acids | 184 |
| 1,12-Bis(trimethylammonium)dodecyl bromide | | | | |
| Tetramethyl (or *n*-butyl)ammonium halide | | | | |
| *(d) Inorganic salts* | | | | |
| Chloride, bromide as alkali metal salts | Silica 60 $F_{254}$ (L); silica 60 $F_{254}$ (C) | (a) Methanol with or without ion-pairing agent; (b) chloroform–methanol (9:1) | Basic drugs | 185 |

[a] (L) = laboratory-made; (C) = commercially available.

### 4.1. *Parameters affecting the retention of analytes*

A number of parameters affecting the retention of the analytes were investigated: thin-layer material, concentration and characteristics of the IP reagent (hydrophobicity related to the chain length of its alkyl groups), organic modifier to water ratio and apparent pH and ionic strength of the eluents[154,155,157–159,161–164,171,174,175,179,182,183,185].

4.1.1. *Layer material on the plates.* As regards the thin layers, commercial alkyl-bonded silica gel plates were generally used and were found to have many advantages over the previously employed laboratory-made plates (considerable decrease in the migration time, greater sensitivity, ability to separate a larger number of compounds in a single chromatographic run)[163,165]. The retention increased substantially from RP-$C_2$ to RP-$C_8$, but varied very little or remained constant on changing from RP-$C_8$ to RP-$C_{18}$[163,170,179]. By impregnating and using under the same experimental conditions bare silica gel commercial plates, obtained from various sources, differences in selectivity were found[174], as reported for unimpregnated silica gel layers[186], showing that the different adsorptive characteristics of silica gel can also affect the retention after impregnation.

4.1.2. *Ion-pairing reagents.* It can be seen from Table 1 that with respect to the inorganic IP reagents, organic counter ions differing in lipophilic character and structure are generally preferred. As regards the influence of the lipophilic character of IP agents, Gonnet *et al.*[170] found a drastic increase in the retention of some dyes with increase in the alkyl chain length of the counter ions tested ($R_F = 1$ with tetrabutylammonium and $R_F = 0$ with tetradodecylammonium). Accordingly, Lewis and Wilson[175] noted an increase in the retention of some hydroxybenzoic acids on RP plates impregnated with long-chain IP reagents such as cetrimide, CTA and DTA with

respect to that observed on layers impregnated with tetramethyl- tetrapropyl- or tetrabutylammonium halides. With inorganic counter ions, chloride, bromide, iodide and perchlorate were found to be efficient IP reagents, whereas sulphate, nitrate and acetate did not give adequate ion pairs. Therefore, differences in retention were obtained by using choride or bromide as counter ions[185].

The structure of the IP reagents must also be considered. Some primary aliphatic amines were retained more strongly on triethanolamine–dodecylbenzenesulphonate- than on sodium lauryl ether sulphate-impregnated RP layers, whereas the retention sequences were the same[154]. As in HPLC[150–152], increasing the concentration of the IP reagent in the plates increases retention until, generally, a limiting value is reached[154,170,174,175]. The optimal concentration of IP reagent solution was found to be 2–4%.

4.1.3. *Organic modifier.* In IP-TLC with RP systems, methanol was generally employed as the organic modifier. No significant change in selectivity was noted on replacing methanol with some other solvents such as ethanol, *n*-propanol, tetrahydrofuran or acetonitrile[160,175]. An increase in methanol concentration resulted in a decrease in the retention of the analytes[154–156,158,160,162,164,170,171,174,175,179], in agreement with that predicted from RP chromatography. Generally, the strongest resolving power of the layers was achieved with 35–50% methanol in the eluent.

A different behaviour was noted with two new bifunctional IP reagents 1,12-bis(trimethylammonium)dodecyl and 1,3-bis(trimethylammonium)propyl bromide. On bare or paraffin-coated silica gel plates impregnated with these reagents, a decrease in retention and a reversal of the retention sequence of the analytes was noted at high methanol concentrations (>80%). This effect might reflect a change in the retention mechanism from reversed to normal phase and can present a potentially useful property of these recently synthesized IP reagents[184].

4.1.4. *Eluent pH.* An important parameter in IP chromatography is the pH of the eluent, as maximum retention is obtained when analytes and IP reagents are completely ionized. When using strongly ionized pairing ions a pH change affects the retention by altering the ionization of the analytes. Compared with columns, where in order to avoid technical problem in IP-RP systems the pH is generally chosen in the range 2–8[152], thin layers are less affected by changes in the pH of the eluent and IP-RP-TLC in the pH range 0.7–11.3 has been reported[159,161,182]. In particular, attention was paid to the general problem of the eluent pH with respect to both the characteristics of the analytes[154,158–163,165,185] and the IP reagents[175,182,183]. As regards nitrogen compounds, on layers impregnated with anionic IP reagents, such as DBS or HDBS, the retention of primary aliphatic and aromatic amines generally decreased on using eluents containing increasing concentrations of hydrochloric acid[154,155,158,165]. With amino acids and peptides, on the basis of the acid–base characteristics of these compounds, species with different charges may exist in solution, depending on the apparent pH of the eluent. Different $R_F$ values were found by Lepri *et al.*[159,160,162,163] for the anionic, zwitterionic and cationic forms of these compounds. For the amino acids investigated, on HDBS-impregnated layers the change in the apparent pH of the eluent was found to be useful for analytical purposes only in acidic media (pH 0.7–6.1); with basic eluents the amino acids were only slightly retained and tailed spots were observed[160].

For some polypeptides, by using plates impregnated with a cationic IP reagent

such as N-DPC, an increase in the apparent eluent pH resulted in a considerable decrease in their $R_F$ values and, for several angiotensins, in a different order of retention. Therefore, an increase in the ionic strength of the eluent caused an increase in retention[164]. For numerous alkyl- and halogen-substituted phenols on N-DPC impregnated layers, as the apparent pH of the eluent was increased, a decrease and a levelling of their $R_F$ values was observed, whereas on layers impregnated with DBS an increase in their $R_F$ values was found with pH values increasing from 5.0 to 11.3[161].

Wilson and co-workers[175,182,183], using bare, paraffin-coated and $C_{18}$-bonded silica gel layers, studied the influence of the eluent pH on the chromatographic behaviour of some hydroxybenzoic acids in relation to the IP reagent employed. Quaternary ammonium halides [$^+NR_4$ and $^+N(CH_3)_3R$] differing in alkyl chain length were investigated. The IP reagents examined fell into two distinct classes: tetrabutyl-, tetraheptyl- and hexyltrimethylammonium halides did not show pH dependence effects, as no significant variations in the $R_F$ values of the test compounds in the pH range 2–11 were noted; in contrast, IP reagents with long lipophilic alkyl chains, such as octyl-, decyl-, tetradecyl- and cetyltrimetylammonium halides, showed a clear pH dependence, with a general increase in the retention as the pH increased. The largest changes in the $R_F$ values were noted in the pH range 2–4.

To establish whether a pH-dependent IP reagent would have only a long alkyl chain, or both hydrophobic and hydrophilic groups such as cetrimide, Troke and Wilson[183] synthesized a bifunctional IP reagent, 1,12-bis(trimethylammonium)dodecyl dibromide, having hydrophilic trimethylammonium groups at each end of the alkyl chain and no hydrophobic end. On paraffin-coated silica gel plates impregnated with this IP reagent, no difference in the retention of the test compounds was obtained over the pH range 2–9, indicating that in the chromatographic system investigated both hydrophilic and hydrophobic groups must be present in the molecule of a pH-dependent IP reagent[183].

In order to understand better the influence of eluent pH on IP liquid chromatography, control, after TLC, of the pH of the whole layer, as reported for other IP reagents[155], and further investigations by using other test compounds differing in p$K$ values and chemical structure, alkyl-bonded silica gel layers instead of paraffin-coated plates and, if possible, comparison with HPLC results, would be useful.

As regards the mechanism operating in IP-TLC, at present the results are not sufficient to establish whether the models proposed for IP-HPLC can be applied to IP-TLC. With surface-active agents as counter ions, the charge of the surfactant ionized groups sorbed on the layers plays an important role in the separation process and an ion-exchange mechanism is hypothesized to be prevalent in many instances[155,158,163,165,168,173,187].

From the results reported in the papers listed in Table 1, some conclusions can be drawn about the usefulness of IP-TLC:

(a) In analytical applications, aliphatic monoamines[154,157], closely related peptides[160,162,172], diastereomeric di- and tripeptides[168], phenols[161,166] and food dyes[156,164,171] have been separated. The use of IP-TLC has been found to have great potential as a general screening method for basic drug analysis, in combination with a general basic development system[185].

(b) Data obtained in high-performance or over-pressurized TLC (HPTLC and

OPTLC, respectively) by using IP-reagent-impregnated layers, could be advantageously applied in HPLC. A good correlation between HPTLC or OPTLC retention values and the capacity factor obtained in HPLC was obtained[170,181]. Therefore, data transfer from HPTLC to HPLC was found to be easier in IP than in RP chromatography[170].

(c) Qualitative IP-TLC has been used for the identification of ionogenic groups in numerous aromatic and aliphatic compounds and it is believed to be a helpful technique in the identification of functional groups of unknown compounds[180].

In conclusion, the above results show that IP-TLC is a very promising, versatile technique and more attention should be paid to its possibilities in future, considering also the increasing use of IP systems in column HPLC.

## 5. PARTITION FLAT-BED CHROMATOGRAPHY ON IMPREGNATED LAYERS

Impregnation of paper or silica gel layers with reagents of low volatility and polarity, such as paraffin or silicone oil, and elution with aqueous–organic mixtures are commonly used in partition reversed-phase chromatography[7,8]. In spite of the great possibilities of chemically bonded silica gel precoated plates[5] in RP-TLC, impregnated layers are preferred by many workers. Normal-phase (NP) partition systems prepared by coating the support, generally cellulose, with polar compounds such as formamide, are also used. Both RP and NP chromatographic systems are utilized for investigations of the molecular lipophilicity of organic compounds and also for analytical purposes.

### 5.1. *Molecular lipophilicity determination: correlation with chemical structure and biological activity*

Lipophilicity, that is the tendency for a species to be readily soluble in most non-polar solvents but only sparingly soluble in water[188], is a useful and important physico-chemical parameter for studies of distribution processes of organic compounds in aqueous media, *e.g.*, penetration through membranes of living cells, bio-concentration in aquatic animals and soil sorption phenomena[189] and more recently to investigate the stability of inclusion complexes[93–101]. Hydrophobicity can be measured in many ways, *e.g.*, by partition between an immiscible polar and non-polar solvent pair (log $P$ values)[190–192], by partition flat-bed chromatography ($R_M$ values)[193,194] and by RP-HPLC (log $k'$ values)[195]. The partition coefficient, $P$, is generally measured in the reference system *n*-octanol–water and it is frequently used to interpret quantitative structure–activity relationships of drugs (QSAR studies). QSAR methods are very useful and widely used in the design of new and biologically effective molecules. The determination of log $P$ values by the usual shake-flask methods is time consuming and often difficult in comparison with the simpler and faster chromatographic techniques. In particular, flat-bed chromatography allows the analysis of compounds available only in small amounts, containing impurities and liable to decomposition. The theoretical basis of the correlation between the chemical structure of a compound and its $R_M$ value obtained from flat-bed liquid–liquid partition chromatography was elaborated by Martin and co-workers[196,197] and experimentally established by Bate-Smith and Westall[198], who introduced the term $R_M = \log [(1/R_F - 1]$, and by Boyce and Milborrow[194]. The latter workers showed that the $R_M$ values

could be used as hydrophobic parameters and that the change in the $R_M$ values for a substituent group ($\Delta R_M$) is a free-energy constant equivalent to the substituent constant $\pi$ obtained from partition data used by Hansch and Leo[199] in QSAR studies. The equation mainly used for the transfer of TLC data:

$$\log P = a R_M + b$$

is an extension of the Collander[190] equation:

$$\log P_1 = a \log P_2 + b$$

where $P_1$ and $P_2$ are partition coefficients in solvent systems 1 and 2, respectively. Naturally, the correlation between log $P$ and $R_M$ values is valid for chromatographic determinations in systems (RP or NP) where partition is either the sole process taking place or predominates over others. In addition, in order to obtain sufficient accuracy, the chromatographic system must give $R_F$ values between 0.2 and 0.8[200,201]. With compounds with large differences in lipophilicity, two or three eluent mixtures, with different organic modifiers, might provide this range of $R_F$ values for all the compounds. $R_F$ values smaller or greater than 0.5 correspond to positive or negative $R_M$ values, respectively. Higher and/or positive $R_M$ values indicate compounds more lipophilic than those with lower and/or negative $R_M$ values. A detailed discussion of the measurement of chromatographic parameters and of the correlations of $R_M$ values with biological and biochemical systems was given in Tomlinson's review[200] and recent achievements in the field can be found in the extensive reviews by Kaliszan[201,202].

Although investigations on chemical structure–chromatographic behaviour relationships[203–211] or QSAR studies[212–222], using the $R_M$ values obtained from partition TLC on plates impregnated with non-polar or polar compounds to express the lipophilic character of the molecules, have been carried out by many workers, in recent years attention has mainly been focused on the chromatographic parameters that could influence the determination of $R_M$ values, aiming always to increase the accuracy of investigations in QSAR studies.

Hydrophobicity can be expressed in terms of solvent partition values either from experiments or by calculation from fragmental constants (Hansch and Leo[199] or Rekker methods). Rekker and co-workers[223,224] reported data obtained from a series of benzophenones using RP-TLC on silica gel plates coated with non-polar compounds. The $R_M$ values correlated well with $\Sigma f$, where $\Sigma f$ is the sum of the hydrophobic fragmental values of the constituent parts of the benzophenone concerned.

As regards the correlations of chemical structure with chromatographic behaviour in RP-TLC systems, a linear relationship between the $R_M$ values and the number of carbon atoms was found by Prandi[203] for numerous aliphatic amines, by Horna *et al.*[206] for alkyl acrylates and methacrylates and by Zemanová and Zeman[204] for N-mono- and N,N-dialkyl-substituted di- and trinitroanilines, in agreement with Boyce and Milborrow's results[194]. For amines, with an increase in the number of functional groups ($NH_2$ or OH) the $R_M$ values increased, whereas a decrease occurred on replacing the amino hydrogen atoms with either alkyl or hydroxyalkyl groups. Śliwiok and co-workers[208,209], using adsorption and partition TLC, compared the

hydrophobic properties of selected isomeric $\alpha$ and $\beta$ derivatives of naphthalene and *cis–trans* geometric isomers (oleic and elaidic acid, respectively, and their methyl esters). Under the experimental conditions tested, adsorption chromatography seemed to be the most efficient method for describing semi-quantitatively the hydrophobic properties of the isomers. The influence of five structural features of alkylphenoxyalkanoic acids on the chromatographic behaviour was investigated by Davídková and Gasparič[211] and the relationhips between chromatographic properties, partition data and chemical structure of a series of O-alkyl-O-arylphenylphosphonothioates were reported by Steurbaut *et al.* [205].

In QSAR studies, Biagi *et al.*[212], one of the first research groups in the field, found a very good correlation between the extrapolated $R_M$ values of a series of alkyl-2-naphthols, naphthols and acetophenones and their antibacterial or haemolytic activity. It should be noted that they used extrapolated $R_M$ values[225]. By extrapolation from the linear part of the graph obtained by plotting the $R_M$ values *versus* percentage of organic modifier in the eluent, the theoretical $R_M$ value ($R_{M_0}$) for each compound at 0% of organic modifier could be calculated. The $R_{M_0}$ value, which should be related to the partitioning of the compounds between water and silicone or paraffin oil, is theoretically independent of the mobile phase composition and $R_M$ or $\Delta R_M$ values obtained in different eluents[225] could be compared. Relationships between $R_M$ and log $P$ values for a series of benzodiazepines[213], 5-nitroimidazoles[214] and xanthone derivatives[216], between $R_M$ or log $k'$ (from HPLC data) and $\pi$ values for a series of potentially mutagenic nitroimidazo[2,1-*b*]thiazoles[215] were reported. In addition, molecular lipophilicity–activity correlations have been formulated for the activity of benzodiazepines in rats[213] and of xanthone derivatives in mice[216]. A linear relationship between $R_M$ values and log $P$ or $\pi$ values was found by Bachratá *et al.*[220] and by Dadáková *et al.*[221] in QSAR investigations on basic esters of substituted carbanilic acids[220] and on crotolactones[221], compounds with potential local anaesthetic and antituberculotic activity, respectively. A correlation between the $R_M$ values and the pharmacological characteristic, log $U$, was established only for the carbanilic acid derivatives. However, the antituberculotic activity of crotolactones is related to a greater number of molecular parameters than simply the hydrophobic properties of the molecules[221].

### 5.2. *Parameters affecting the determination of $R_M$ values*

Many parameters can influence the determination of $R_M$ values, *e.g.*, the layer material, the impregnating agent used as the stationary phase and its concentration, the composition of the mobile phase and the chemical structure of the compounds.

The layer material may partially retain its original adsorptive characteristics even after impregnation, as reported by many workers[211,214–216,221–231]. Gasparič[226], in a study on the use of RP-TLC to determine molecular lipophilicity, investigated the chromatographic behaviour of compounds differing in polarity. Cellulose or silica gel plates and liquid paraffin or 1-octanol as impregnating agents were tested. The hydrophilic compounds behaved as if the layers were not impregnated, whereas the strongly lipophilic species followed the RP partition mechanism. A comparison of the adsorptive behaviour in RP-TLC of Kieselguhr, cellulose, Kieselgel and silanized Kieselguhr plates, all impregnated with oleyl alcohol, was reported by Van der Giesen and Janssen[227]. Kieselguhr and cellulose showed

adsorption, whereas Kieselgel is not a good support, probably because of its large specific surface area. Silanized Kieselguhr gave a support without adsorptive sites; the $R_M$ values obtained correlated very well with log $P$ values. By using paraffin oil-coated silica or alumina plates, different $R_M$ values and different dependences of retention on the percentage of organic modifier in the eluent were obtained by Cserháti[228] for some 3,5-dinitrobenzoic acid esters. Silica, alumina and celluose plates coated with paraffin oil were compared by Cserháti *et al.*[229] for the determination of the lipophilicity of some neutral, acidic and basic compounds. In the RP-TLC experiments, basic compounds showed higher $R_M$ values on silica and acidic compounds on alumina, indicating that with polar compounds the surface pH of the support, also after impregnation, influenced the determination of lipophilicity. In measurements of the lipophilicity of benzophenones, Bijloo and Rekker[224] investigated the influence of modifications of the stationary phase by using different layer materials (silica gel or silica gel–Kieselguhr mixtures) and different coating agents (paraffin or silicone oil). The replacement of paraffin with silicone oil caused significant changes in the retention of compounds with particular functional groups and made it difficult to explain the investigated structures in terms of lipophilicity. The addition of Kieselguhr to the silica influenced only the behaviour of the silicone oil-coated plates.

Silicone and paraffin oil are widely used as non-polar stationary phases and a concentration range of 5–10% generally permits the required separation. However, lower impregnation (<2%) improved the separation of organophosphate esters by RP-TLC in both chromatographic systems tested by Gandhe *et al.*[232]. In undecane, squalane or liquid paraffin systems, the $R_M$ values of 5-nitroimidazoles where higher than in a silicone oil system[214]. Differences were found between silicone oil and 1-octanol[213] and between paraffin oil and a series of $C_{16}$, $C_{18}$ and $C_{20}$ fatty acids or their esters as stationary phases[218]. By using secondary amide derivatives, better reproducibility of the $R_F$ values was observed by Churáček[233] on formamide- than on dimethylformamide-coated layers, and for some triazine herbicides a linear relationship between the $R_M$ values determined by using silanized plates coated with two different stationary phases (diethylene glycol and formamide) was found by Ogierman and Silowiecki[234].

The effect of the composition of the eluent on the determination of molecular lipophilicity was also investigated. With compounds containing dissociable substituent groups, the eluent pH[235] and salt concentration can modify the lipophilicity[236,237]. Cserháti and Gasparič[238] showed that when buffer solutions are used as the mobile phase in both NP- and RP-TLC systems in QSAR studies of ionizable compounds, false results could be obtained if the possible change in the support pH, due to eluent demixing and/or to the buffering effect, is not considered. From a comparison of the buffer capacities of sodium phosphate, sodium acetate and sodium diethylbarbiturate (veronal buffer), all at pH 8.5, on paraffin oil–silica gel-coated plates and with methanol–water mixtures as eluents, the movement of the alkaline front decreased with increasing methanol concentration and with increasing extent of coating. The buffering capacity increased in the order veronal (highest $R_F$ value, 0.12) < phosphate (up to $R_F = 0.50$) < acetate. Only the acetate produced a buffering effect that was evenly distributed along the plates.

As regards the effect of different organic modifiers on the determination of molecular lipophilicity[207,210,239–245], aqueous mixtures of acetone, acetonitrile,

TABLE 2
ANALYTICAL SEPARATIONS BY USING PARTITION CHROMATOGRAPHY ON IMPREGNATED LAYERS

| *Compounds or class of compounds* | *Thin layer*[a] | *Impregnating agent* | *Eluent* | *Remarks* | *Ref.* |
|---|---|---|---|---|---|
| Aliphatic amines | Kieselguhr (C) | 5% paraffin oil in acetone | Acetone–17% ammonia (55:45 or 70:30) | Good separation of fatty amines | 203 |
| Barbituric acid derivatives | Silica gel 60H (L) | 5% paraffin oil in benzene–acetone (1:1) | Water–acetonitrile, acetone, isopropanol, methanol in various proportions | Best eluent: water–isopropanol | 207 |
| Alkylphenoxyalkanoic acids | Silica gel and cellulose sheets (C) | (a) 10% liquid paraffin in *n*-hexane; (b) 20% formamide in methanol or 50% DMF in acetone | Various | On formamide layers higher $\Delta R_M$ values for $CH_2$ group of all the homologues | 211 |
| Diethylphenylphosphates | Silica gel G (L) | (a) 1.5% *n*-octanol in hexane; (b) 1.5% silicone oil in ethanol | Acetone–water (40:60) | Separation of positional isomers with both the RP-TLC systems | 232 |
| Triazine herbicides | Silica gel G (L) | (a) 20% diethylene glycol in acetone; (b) 20% formamide in acetone | (a) *n*-Hexane–benzene–THF (4:1:1); (b) *n*-hexane–chloroform–diethyl ether | Separation of most herbicides in both TLC systems | 234 |
| Aliphatic $C_2$–$C_{18}$ alkyl esters of acrylic and methacrylic acids | Silica gel (L) | 5 or 10% paraffin oil in light petroleum | (a) DMF–methanol–water (9:5:1); (b) DMF–water in various ratios | No derivatizion of the compounds before TLC. Poor separation of some neighbouring members in each series | 206,247 |

| | | | | | |
|---|---|---|---|---|---|
| $C_2$–$C_{18}$ alkyl esters of acrylic acid | Silica gel (C) and cellulose (C) | (a) 5% paraffin oil in light petroleum; (b) 40% DMF in ethanol | (a) DMF–methanol–water (2:1:1); (b) cyclohexane–benzene | Derivatization with diazomethane prior to TLC. In the RP-TLC system (a), complete separation of the whole homologous series | 248 |
| $C_{19}$ steroids | Cellulose (C) | 1,2-Propanediol in methanol | Benzene–cyclohexane (50:50) | Separation of various 3-hydroxy epimers of $C_{19}$ steroids, such as androsterone and epiandrosterone | 246 |
| Hydroxycinnamic acids | Silica gel 60 H (L) | 5% paraffin oil in benzene–acetone (1:1) | Different buffers at various pH (2.9–10.1) | Separation of *cis–trans* isomers in the pH range 4.5–6.5 | 249 |
| Organophosphorus insecticides | Silica and alumina sheets (C) | 60% DMF in methanol | DMF saturated with *n*-hexane | Separation of Phoxim from Baytex from human cadaver organs | 252 |
| Ecdysteroids (insect-moulting hormones) | Silica gel (C) | 7.5% Nujol in dichloromethane | Methanol–water (1:1) | For the separation of this class of compounds, both NP- and RP-TLC systems can be used | 255 |
| Dothistromin (metabolite produced by *Dothistroma pini*) | Silica gel $GF_{254}$ (L) | 5% paraffin oil in hexane | Methanol–water (2:1) containing 4% formic acid | Quantitative analysis after separation from chlorophylls and other compounds | 250 |
| Reserpine and ajmaline in *Rauwolfia vomitoria* | Silica gel (C) | 20% formamide in acetone | Methyl ethyl ketone–*n*-heptane (1:1) in ammonia atmosphere | Determination of both alkaloids | 251 |
| Steroidal glucosiduronic esters | Paper | Formamide | Various liquid ion exchangers in cloroform | Resolving characteristics of the various systems compared in terms of $R_M$ values | 144 |

[a] (L) = laboratory made; (C) = commercially available.

methanol and tetrahydrofuran[210] or isopropanol[207] were investigated. For phenolic[210] and barbituric acid[207] and aniline derivatives[243], linear relationships between $R_M$ values and the percentage of the organic modifier were observed for most of the compounds investigated. However, the $R_M$ values of the aniline derivatives were lowest in acetonitrile and highest in methanol. The solvent strength was acetonitrile > acetone > methanol[243]. Using a spectral mapping technique, Cserháti and Bordás[240] evaluated the relative strengths of 27 organic solvents in RP-TLC on impregnated layers. In determinations of the lipophilicity of *n*-alkyl phenyl ketone homologues, acetone appeared to be less suitable than methanol or N,N-dimethylformamide, probably because of acetone-induced perturbations of the stationary phase[241]. The use of a hydrophobic parameter on the basis of $R_M$ values, but independent of the composition of the mobile phase, was suggested by Draffehn *et al.*[242]. A comparison of extrapolated $R_M$ values ($R_{M_0}$) with water, aqueous methanol or aqueous acetone as eluents was reported by Barbaro and co-workers. Silica gel plates coated with silicone oil as stationary phase and dermophin-related oligopeptides[244] or a series of prostaglandines[245] as test compounds were examined. It was pointed out that the relationship between $R_M$ values and the percentage of organic modifier can generally be described by an S-shaped curve. They suggested calculating the $R_{M_0}$ values from the linear part of the curve, which corresponds to a narrow range of organic solvent concentration. In this way, very similar $R_{M_0}$ values were obtained from both the water–acetone and water–methanol systems, confirming that the extrapolated $R_{M_0}$ values are independent of the nature of the organic modifier[225]. For some of the test compounds, the $R_{M_0}$ values were very similar to the experimental $R_M$ values obtained with water as the eluent[244]. The data seem to provide a further contribution to the use of RP-TLC as a standard system for molecular lipophilicity determinations.

5.3. *Analytical separations*

In Table 2 the compounds or classes of compounds investigated for analytical purposes by partition TLC on coated plates, the chromatographic systems used and the results obtained are reported. A wide variety of compounds have been analysed with good results, which in many instances cannot be obtained on bare silica gel plates[206,207,232,246]. As can be seen, both RP[203,206,207,211,242,247–250] and NP[211,234,246,248,251,252] partition TLC were employed. A systematic collection of data on the chromatographic behaviour of a large number of aliphatic amines, with particular emphasis on eluents, $R_F$ values and detection reagents, was reported by Prandi[203]. From these data, relationships between chemical structure, chromatographic behaviour and physical properties of the amines were obtained, which may be useful for identification purposes.

Some quantitative determinations have been reported[250,251]. With dothistromin, the sole metabolite identified in extracts of diseased pine foliage, the time required for sample application, TLC and analysis was 2 h. The method proposed may also be useful for the examination of foliage polyphenols and phytoalexins, where interference from chlorophylls may be a problem[250].

5.4. *Chemically modified and impregnated plates: comparison for reversed-phase thin-layer chromatographic use*

RP-TLC on alkyl-bonded silica gel plates has become widely used in the last 10 years[5] and has superseded the earlier use of paraffin- and silicone-oil-coated silica gel layers. A comparison between the two types of RP-TLC plates may help in the choice of the best system in relation to economy, convenience or saving of time.

In a series of studies[253–255], Wilson and co-workers compared $C_8$, $C_{12}$ and $C_{18}$ materials bonded with analytical and preparative paraffin-coated silica gel plates using a wide range of organic test compounds. In order to obtain comparable results, coated and bonded types were chromatographed simultaneously in the same TLC tank. On both paraffin-treated (analytical and preparative) and OPTI-UP-$C_{12}$ (ref. 253) or on non-hydrophobic bonded $C_{18}$[255] a similar order of retention of ecdysteroids was found. As regards quantitative determination, comparable results were obtained on all three types of layers for amounts of material of the order of 200 ng, whereas below 100 ng per plate $C_{12}$ gave better results[242]. In all instances, the $C_{18}$ layers required a higher concentration of methanol in the eluent to achieve the same $R_F$ values as on the coated plates. Like the non-hydrophobic $C_{18}$ bonded plates, the paraffin-coated plates could be used with solvent systems containing from 0 to 100% of water[253–255].

Correlation plots of $\log k'$ (from HPLC results on $C_2$ bonded silica gel) *versus* the $R_M$ values obtained on silanized and paraffin-impregnated silica gel layers were reported for some phenolic acid derivatives by Grodzińska-Zachwieja *et al.*[210]. The correlation was better for the silanized plates. However, the identical retention sequences of the test compounds on both bonded and coated plates compared with those obtained on a column indicated a similar separation mechanism and proved the usefulness of RP-TLC systems. As reported in Table 2, plates coated with formamide and liquid paraffin and RP-2 and RP-18 precoated HPTLC plates were compared for separations of alkylphenoxyalkanoic acids by Davídková and Gasparič[211]. RP-HPTLC on precoated plates and NP-TLC on formamide-coated silica gel layers seemed to be the most efficient methods.

A simple method of two-dimensional TLC with different separation mechanisms in the two directions was proposed by Wilson[256]. Normally, for this type of two-dimensional TLC commercially available RP-18 plates, which have a narrow strip of bare silica gel, are used. As an alternative, a section of a silica gel TLC plate may be silanized in the laboratory. The method proposed by Wilson is based on the incorporation of paraffin in the normal-phase solvent system used to obtain the initial separation. During the development in the first direction, a concomitant coating of the layer is obtained, and after the evaporation of the solvent a TLC plate ready for subsequent RP-TLC in the second direction is obtained. Of the numerous compounds tested, only with ecdysteroids was it not possible to add paraffin to the first solvent system. In such instances it may be possible to coat the TLC plates after the normal-phase separation.

In conclusion, partition TLC on plates coated with very low or very high polarity materials has been succesfully used in molecular lipophilicity determinations and for analytical purposes. For most applications, with appropriate modifications to the eluent paraffin-coated and non-hydrophobic $C_{18}$ bonded silica gel plates may be interchangeable[255] and could be used for the optimization of the separation conditions in column HPLC[210]. In addition, whereas paraffin-coated plates can be used with any

proportion of water in the eluent, many types of commercially available silanized plates are hydrophobic, with a consequent limitation on the use of water in the eluent and in the direct application of biological samples to the layers[5]. However, in spite of the suggestion by Tomlinson[200] that chromatographically obtained parameters should have wider applicability in structure–activity relationships, papers dealing with the determination of lipophilicity by using TLC on coated or bonded plates are not very numerous. In addition, a comment is needed on some of the reported papers. In some instances the linear relationships reported by plotting $R_M$ values *versus* the percentage of organic modifier in the eluent were obtained from few and/or not always chromatographically significant $R_F$ values ($<0.2$ and $>0.8$). Indeed, for the most retained compounds, a small difference in $R_F$ values (*e.g.*, $R_F = 0.04$ or 0.06) corresponds to a significant difference in $R_M$ values (1.38 and 1.19, respectively). For compounds with $R_F > 0.8$, the uncertainty in the eluent front determination, due to probable solvent demixing of the mobile phase, can cause erroneous results.

## 6. pH CHANGES OF THE LAYERS

In liquid chromatography, the pH of the system exerts a marked effect on the chromatographic behaviour of ionizable compounds. Indeed, as reported by many workers, the retention of the same analyte in the protonated or deprotonated forms may differ considerably. In HPLC, reversed-phase columns can be made to retain weakly acidic or basic samples by buffering the eluents in the pH range 2–5 or 7–8, respectively, that is, by a shift of the chemical equilibria to undissociated forms. This technique has been termed "ion suppression" and a discussion of the current thinking may be found, *e.g.*, in a review by Bidlingmeyer[152]. In flat-bed chromatography, a convenient variation of this approach is to modify the pH of the layer. This treatment before the chromatographic development may be performed with the vapour phase of aqueous acids or bases[257–259] or by dipping the paper[260] or the plates[261–264] in aqueous acids[260,262] or bases[263] or in buffer solutions[261]. By an appropriate change in the pH of the layer, the separation of compounds of similar structure, such as benzoic acid or aniline derivatives[257], polar aromatic compounds[259], coumarin anticoagulants[261], amino acids[262] and barbiturates[263], was improved. Very sharp separations of six noble metals, $Au^{III}$–$Os^{IV}$–$Pt^{IV}$–$Pd^{II}$–$Cu^{II}$–$Ag^{I}$, and inorganic or organic mercury analysis[258] were also performed by Przeszlakowski and Flieger[260] and by Bruno *et al.* [258], respectively, by conditioning the layers prior to elution.

The advantage of the use of pH gradient layers was presented by Stahl and Müller[264] and confirmed by Quirin[265], when tested on crude phospholipid extracts of animal and vegetable origin or on a model mixture of ten components.

## 7. ELECTROLYTE-IMPREGNATED LAYERS

By addition of electrolytes to a chromatographic system, the ionic equilibrium may change and an improvement in the separation process is obtained. In column chromatography, sharp separations have been obtained by adding various electrolytes to the eluents[266], whereas in flat-bed chromatography, salt-impregnated paper or plates are commonly used to improve the separation of a wide range of compounds[8].

Many investigations on the separation of sugar mixtures by means of TLC on electrolyte-impregnated plates have been carried out and were reviewed by Ghebregzabher *et al.*[267]. Silica gel layers impregnated with mono- or dihydrogenphosphates were found to give the best results by Ovodov *et al.*[268]. As the solubility of sugars in inorganic salt solutions is known to increase, within certain limits, with an increase in salt concentration, a higher solubility of the sugar in the stationary phase and a decrease in the $R_F$ values were obtained, with consequently better separations[268]. In recent years, some attempts have been made to improve the TLC separation of carbohydrates using phosphates as impregnating agents[269–272]. The determination of glucose, fructose and sucrose in molasses by HPTLC[270] and the application of sintered silica gel plates[271] or of aminopropyl-bonded silica gel plates[272] instead of plain silica gel plates have been reported. Potassium dihydrogenphosphate-impregnated silica gel plates were also employed by Touchstone *et al.*[273] for the separation of bile acids from diluted specimens of human bile. Potassium oxalate is another salt commonly employed as an impregnating agent. Numerous studies on the metabolism of phosphoinositides in biological membranes by using TLC on potassium oxalate–silica gel plates have been reported[274–280]. Calcium oxalate-impregnated silica gel plates were employed for the separation and identification of closely related sulpha drugs by Srivastava *et al.*[281] whereas in a study of the TLC separation of aromatic amines, the importance of the anion and not of the cation impregnation was indicated[282]. Two-dimensional TLC of polar lipids on ammonium sulphate-impregnated silica gel plates was developed by Jain and Subrahmanyam[283] and various salts were found to be good impregnating agents in the TLC separation of metal ions by Ajmal *et al.*[284].

In conclusion, pH gradient plates, with three different run directions, have not been used as expected, whereas potassium oxalate-impregnated plates are widely employed as sequestrating agents for any calcium that might be present when chromatographing phosphoinositides[8] and in their separation in studies of their metabolism in biological membranes.

## 8. CONCLUSION

As shown in Table 3, many compounds can be analysed by flat-bed chromatography on impregnated layers, achieving the separation by very different retention mechanisms. In most instances, separations not achieved on plain paper or silica gel plates were obtained on impregnated layers and quantitative results were reported. The advantages of this flat-bed technique over HPLC and GC are economy, technical simplicity and the possibility of testing, relatively rapidly, a large number of impregnating agents as possible packing materials for TLC and HPLC. As regards the future, the very promising ion-pair and ligand-exchange techniques, widely used in HPLC, deserve major attention in TLC on impregnated plates. In addition, more investigations on the chromatographic parameters, as performed by only a few research groups, could clarify the mechanisms of the separation processes and make possible further advances in TLC.

TABLE 3
COMPOUNDS OR CLASSES OF COMPOUNDS ANALYSED BY FLAT-BED CHROMATOGRAPHY ON IMPREGNATED LAYERS

| *Compounds or classes of compounds* | *Ref.* | *Compounds or classes of compounds* | *Ref.* |
|---|---|---|---|
| Acetophenones | 212 | β-Cyclodextrins | 120 |
| Hydroxy- | 46 | Dothistromin | 250 |
| Acrylic and methacrylic acids | | Drugs | 55,136 |
| (aliphatic $C_2$–$C_{18}$-alkyl esters) | 206,247,248 | Anti-inflammatory | 254,256 |
| Ajmaline | 251 | Basic | 185 |
| Aliphatic acids (aryl) | 222 | Sulpha | 52 |
| Alkaloids | 51,135,179 | Dyes | 63,65 |
| Amines | 88,92,139 | Azo | 121,220 |
| Aliphatic | 154,158,165, 180,203 | Fluorescent | 264 |
| | | Food | 156,170,171 |
| Aromatic | 155,157,180, 285, 286, 290 | Synthetic | 64 |
| | | Enantiomers | 96–107 |
| Amino acids | 50,57–62,90,92, 134,159,163,165, 169,173,231,235, 237,262 | Fatty acids | |
| | | Cholesteryl esters | 16 |
| | | Isomers | 22–25 |
| | | Labelled methyl esters | 29 |
| | | Polyunsaturated methyl esters | 19,21 |
| ω-Amino acid derivatives | 218 | *trans*-Hexadecenoic and | |
| Amino sugars | 165 | *trans*-Octadecenoic methyl esters | 33 |
| Aniline derivatives | 204,257 | Gibberellins (*p*-nitrobenzyl esters) | 28 |
| Ring-substituted | 236,243 | Glycerides | 30 |
| Anions | 140,141 | Hydrocarbons (polycyclic aromatic) | 49 |
| Antibiotics | | Imidazoles (5-nitro) | 214 |
| 4-Epi-meclocycline | 86 | Indole derivatives | 167 |
| Polymyxin | 115 | Inorganic ions | 70–79,129–133,292 |
| Tetracyclines | 83–85, 87, 291 | Isomers, positional | 113 |
| Barbituric acid derivatives | 66,119,207,263 | Ketones (*n*-alkylphenyl) | 241 |
| Benzodiazepines | 213 | Lactulose | 43 |
| Benzophenones | 223,224 | Lichen acids | 47 |
| Benzoic acid | | Lysophospholipids | 24 |
| Derivatives | 257,259 | Mercaptans | 91 |
| Esters | 228 | Mercury (inorganic and organic) | 258 |
| Hydroxy- | 254 | Naphthalene (α and β derivatives) | 208 |
| Bile acids (dihydroxy conjugated) | 273 | Naphthols | 212 |
| Bufadienolides | 37 | Noble metals | 260 |
| Cannabinoids | 293 | Nucleic acids | 49,50 |
| Carbamates | 53 | Peptides | 160,165,169,172,235 |
| Carbanilic acid derivatives | | Dipeptides | 162,163,168 |
| Basic esters | 220 | Oligopeptides | 244 |
| Carbohydrates | 68,89,271 | (dermophin related) | |
| Cardenolides | 36–38 | Polypeptides | 164 |
| Chelating agents (sulphonated) | 142 | Pesticides (organophosphorous) | 288,289 |
| Cholesterol | 335 | Phenolic acids | 56 |
| Cinnamic acid derivatives | | Polyhydroxy derivatives | 67 |
| Hydroxy- | 210 | Phenols | 138,161,174,287 |
| cis- and *trans*-hydroxy- | 249 | Acid and aldehydes | 54 |
| Copper | 81 | Amino | 254,256,290 |
| Coumarins | 227,261 | Chloro | 99 |
| Crotonolactones | 221 | Chloro, bromo, alkyl | 166 |
| Crown ether derivatives | 230 | | |

TABLE 3 *(continued)*

| *Compounds or classes of compounds* | *Ref.* | *Compounds or classes of compounds* | *Ref.* |
|---|---|---|---|
| Nitro, cyano, halogeno | 229 | Androstanes, androstenes | 246 |
| Phenothiazine bases and sulphoxides | 176 | Ecdysteroids | 253–256 |
| Phenoxyalkanoic acids (alkyl, substituted) | 211 | Steroidal glucosiduronic esters | 144 |
| | | Styrene (nitro-) | 118 |
| Phenylhydrazones (2,4-dinitro-) | 229 | Sugars | 269,270,272 |
| Phosphates (diethylphenyl) | 232 | Sulphonamides | 157,217,219,226 |
| Phosphatides | 266 | Sulphonic acids | 174,180 |
| Phosphoionositides | 40,274–280 | Terpenoids | 18 |
| Phospholipids | 20,30,39,41 | Thiazole derivatives | 215 |
| Phosphonothioates (phenyl) | 205 | Triazines | |
| Prenyllipids | 23 | 17-Substituted symmetric | 240 |
| Prostaglandins | 31,32,245 | Trisubstituted symmetric | 229 |
| Purine derivatives | 48 | *s*-Triazine herbicides | 34,116,234 |
| Pyrimidine derivatives | 48 | Triphenyl methane | 117 |
| Pyrazole derivatives | 45 | Triterpene alcohols (trihydroxy pentacyclic) | 26 |
| Rare earths | 143 | | |
| Resorcinol derivatives | 27 | Uranium | 80 |
| Ribonucleotide reductase | 44 | Xanthone derivatives | 216 |
| Steroids | 82,239,242 | | |

## 9. SUMMARY

It is shown that improvements in paper and TLC analysis can be obtained by impregnating the layers with compounds differing in chromatographic behaviour. The results obtained by using complexing, ion-exchanging and ion-pairing agents are reviewed. Particular attention has been paid to the determination of molecular lipophilicity, as this parameter is widely used in studies in correlations with chemical structure and with biological activity (QSAR). For these studies, layers impregnated with compounds of very low or very high polarity and the appropriate eluents were used. The comparison between chemically modified and impregnated silica gel plates is discussed. Table 3 shows all the compounds or classes of compounds analysed by flat-bed chromatography on impregnated layers.

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