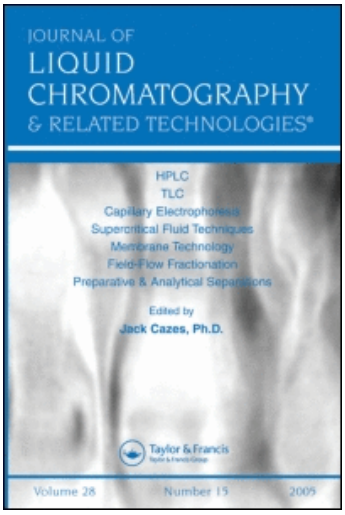

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Haleem J. Issaq[a]
[a] Chemical Carcinogenesis Program NCI Frederick Cancer Research Center, Frederick, MD

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MODIFICATIONS OF ADSORBENT, SAMPLE AND SOLVENT IN THIN LAYER CHROMATOGRAPHY

Haleem J. Issaq
Chemical Carcinogenesis Program
NCI Frederick Cancer Research Center
Frederick, MD 21701

ABSTRACT

This review covers, from a practical point of view, the modifications of the sample, adsorbent and solvent, which are available to achieve the separation of complex chemical mixtures by thin layer chromatography.

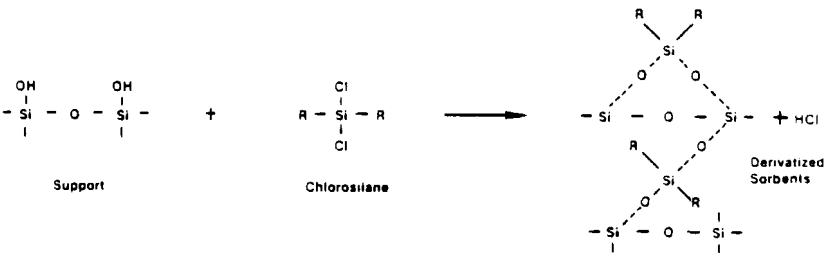
INTRODUCTION

Since thin-layer chromatography (TLC) was introduced as a micro-analytical technique many adsorbents, solvents and developing systems have been developed to assist in the resolution of a wide range of chemical mixture. The adsorbents described include alumina, cellulose, charcoal, polyamide, silica gel, and talc. A layer of mixed adsorbents has also been used. The solvent systems used for developing the chromatograms have been pure solvents (methanol, hexane, ethylacetate, etc.) or a mixture of up to six solvents as needed. A few microliters of the samples dissolved in an appropriate solvent are spotted on to the adsorbent layer. The plate is then developed in a jar or special apparatus (sandwich tank, U-chamber, compressed system, etc). The plate is developed in one or two dimensions or continuously, either at room-temperature or in the cold.

A recent trend has been to modify the properties of the adsorbent, the solvent and the sample. This paper reviews these modifications, comments on them, and suggests examples for their use.

1. ADSORBENT MODIFICATIONS:

a) Chemical: The adsorbent is modified by a chemical reaction by which a group of interest is bonded to the adsorbent to alter its properties. Silica gel layers have been modified by silylation to form a more hydrophobic (lipophilic) phase. Such phases are prepared by bonding an organic group R to the surface of silica to form ≡Si-R or ≡Si-O-Si-R groups according to the following reaction.



By changing the length of the chain, or the group (R), the properties of the resulting derivatized sorbent are changed. Reversed phases bonded with C_2, C_6, C_8, C_{12} and C_{18}, in the bonded group (R), are commercially available. The group itself may also be changed as is the case in high performance liquid chromatography (HPLC) adsorbents. The above derivatization procedures are carried out before a plate is made.

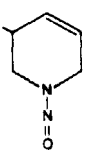
Aringer and Eneroth (1), on the other hand, silylated a commercially available precoated silica gel plate by developing the plate in the very reactive agent hexamethyldisilazane (HMDS). Using derivatized silica gel as modified adsorbent for TLC allows the migration and separation of polar sample components which would otherwise bind strongly to silica gel. The

order of migration of the compounds on bonded silica gel phases is the reverse of that on silica gel phases. Such bonded phases are, therefore, referred to as reversed phases. Numerous examples are cited in the literature. A few will be given here. Environmental trace analyses were carried out on C_8 and C_{18} reversed phase plates (2). A mixture of 1-, 2-, and 4-acetylaminofluorene isomers was resolved on reversed phase C_{18} plates but not on silica gel (3). Brinkman and DeVries (4) separated a mixture of dialkylpthalate on C_8 reversed phase plates. A comparison between the resolving power of silica gel, silanized silica gel (HMDS) and C_{18} reversed phase silica gel plates indicated that the latter gave the best resolution of the oxidation products of cholesterol (3). Van Arx and Faupel (5) separated mixtures of steroids and peptides on reversed phase silica gel and alumina plates which they had prepared. A theoretical treatment of the separation on reversed phase and normal phases was published by Martire and Boehm (6).

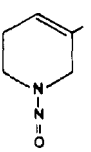
b) Impregnation: There are different methods for impregnating the adsorbent depending on whether the plates are prepared in the lab or are precoated by the manufacturer. For plates that are prepared (in house) the reagents are added to the slurry. This method gives uniform and controlled concentrations of the reagent. If precoated plates are used, the reagent is introduced by spraying (not very uniform), dipping, developing the plate in the reagent solution or by brushing the reagent solution on the plate. A discussion of impregnation was presented by Halpaap and Ripphahn (7). The adsorbent is normally impregnated with a reagent that forms a complex or an addition compound with the component mixture. It is a selective process where one component reacts with the reagent while the other does not whereby separation is achieved. For example, boric acid is used for the separation of vicinal dihydroxy isomers where a cyclic boric acid derivative is formed; silver ions form complexes with olefinic double bonds and so cis- and trans- isomers can be separated; metal ions

such as zinc, cadmium, manganese, etc. form complexes with nitrogenous bases; picric acid and trinitrobenzene form complexes with polynuclear compounds. Halpaap and Ripphahn (7) studied the effect of impregnation of hydrophilic and lipophilic stationary phases on R_f values. Stable hydrophilic stationary phases were formed by impregnating precoated silica gel plates with formamide, dimethyl formamide, ethylene glycol, polyethylene glycols, 2-phenoxyethanol and various buffers. Lipophilic stationary phases (for reversed phase chromatography) were obtained by impregnating the silica gel plates with mineral oils, liquid paraffin, undecane, silicone oils, tetradecane or ethyl oleate. They found that if higher R_f values are required, the hydrophilic component of the solvent must be increased in the case of hydrophilic adsorbents, while the lipophilic solvent component must be increased when lipophilic adsorbents are used (7). Touchstone et al (8) separated a mixture of dihydroxy bile acids on potassium dihydrogen phosphate-impregnated silica gel plates.

Impregnation of plates with formamide (9) and boric acid (10) for the separation of isomers of cardenolides were also reported. The separation of N-nitroso-3-methyl Δ^3-tetrahydropyridine from N-nitroso-5-methyl Δ^3-tetrahydropyridine was achieved on silver nitrate impregnated silica gel plates



N-nitroso-5-methyl- Δ^3-tetrahydropyridine



N-nitroso-3-methyl - Δ^3-tetrahydropyridine

by forming a complex between the silver ions and the π-electrons of the double bond on N-nitroso-5-methyl-Δ^3-tetrahydropyridine and not the 3-methyl-isomer (11). Methylesters of polyunsaturated fatty acids were also separated by impregnating the plate with silver ions (12).

Other metal ions were used, for example, Martz and Krivis (13) sprayed silicic sheet plates with cooper sulphate solution before spotting to obtain separation of hexosamines and n-acetylhexosamines. Our experience indicates that more uniform impregnation is achieved when

precoated plates are dipped or developed in the reagent solution than when sprayed (14).

Yasuda used silica gel plates impregnated with cadmium sulfate (15) cadmium acetate (16) and manganese salts (17) to separate mixtures of aromatic amines. Silica gel and alumina plates impregnated with zinc salts (18,19) calcium oxide (18) and cadmium nitrate (19) were used for various separations including a mixture of nine toxic alkaloids which did not separate on silica gel or alumina (19).

Antonelli et al (20) used ligand exchange TLC to separate a mixture of α-aminoacids, their β-isomers and peptides. The chelating exchangers used were $Cu(NH_3)_x^{2+}$ on which α-amino acids were retained, and $Ni(NH_3)_6^{2+}$ which gave better separation of the above three groups of compounds. Ligand Exchange Chromatography (LEC) is a very promising technique. It has been defined (21), "as a process in which interaction between the stationary phase and the molecules to be separated occurs during the formation of coordination bonds inside the coordination sphere of the complex forming ion". Davankor and Semechkin (21) have published a comprehensive review of LEC.

Grant and Meiris (22) mixed Bentone-34, dimethyldioctadecyl ammonium bentonite, with silica gel for the selective separation of some polycyclic aromatic hydrocarbons. They concluded that better separations were achieved with the mixed phase plate than with silica gel plates. Mixtures of nucleosides, nucleotides, and nucleic acid bases were separated on microcrystalline cellulose layers mixed with chitosan, deacetylated chitin, 2:1 by weight (23).

Soap TLC (24) consists of mixing a detergent with silanized silica gel during the slurry formation. The detergents used were sodium laurylether osulphate, triethanolamine dodecylbenzenesulfonate, and sodium dodecylhydrogensulfate. The plates were used for the separation of some aliphatic amines. The effect of concentration of detergents on separation and R_f values were also studied and compared with that on layers of strong

and weak anion and cation exchangers (25). It was found that the separation of the amines is due to a partition process between the two phases and not due to an ion exchange mechanism.

It is important to note that when silica gel layers are modified with hydrophilic or lipophilic reagents, the separation process is changed from adsorption to partition chromatography. It is also worth remembering that impregnation of the adsorbent is a selective process and its use depends on the properties of the sample. Impregnation is not limited to silica gel, other adsorbents such as alumina or cellulose may be modified.

Gradient layers have been discussed by Stahl (26). Recently pH gradient layers were used to separate a mixture of fluorescent dyes (27). These layers are also recommended for the separation of mixtures of compounds of widely differing polarities. Gradient layers with increasing impregnation of silver nitrate were reported (28,29).

Complexing agents are added to the adsorbent not only for separation purposes but for visualization and identification. Some chromagenic reagents based on the formation of complexes between Fe^{+3} ion and organic acids have been reported (30,31). The formation of blue color complexes between Cu^{+2} ion and some polybasic acids has been used for the densitometric quantitation of the resulting compounds (32).

2. SAMPLE MODIFICATIONS:

a) In Solution: Derivatization of the sample mixture to separate closely related components has long been used in gas liquid chromatography (GLC) and recently in HPLC. However, the use of derivatization in TLC, as an aid in the separation of a mixture, has not been fully exploited. Unlike GLC, where derivatization is used mainly to form volatile, thermally stable compounds, and HPLC where derivatization is used to introduce a chromaphore, in TLC it is used to accentuate structural difference by introducing "bulky" groups into the sample mixture; make separation possible. Also, by steric hindrances and polarity strength changes.

Separation of the two isomers of 3-hydroxy-N-nitrosopiperidine was achieved after derivatization in solution with TSIM (11). Also a mixture of oxidation products of cholesterols were separated on silica gel plates after derivatization with TSIM, while separation was not possible without derivatization (33).

b) On the Plate: Hwang et al (34) combined the Schiff's base reaction characteristics with TLC to determine primary amines. The sample is spotted on the plate followed by an addition of an equal volume of 1-pyrene aldehyde in ethanol after which the plate is sprayed with 1-butanol acidified with dry hydrochloric acid and placed in an oven for 1 hour at 70°C, then developed. Schutz (35) used reaction on the plate to separate and identify nitrazepam and its metabolites. After separation in the first dimension of two-dimensional thin-layer chromatography, the substances are derivatized by treatment with an acidified aqueous solution of $TiCl_3$. By means of combined hydrolysis and reduction nitrazepam and its metabolites are converted on the plate to reproducible derivatives. The subsequent separation in the second dimension permits exact identification from the R_f values and Bratton-Marshall detection.

In another study (36) Soyasapogenols A, B, C, D and E were separated on silica gel plates after derivatization with acetic anhydride:pyridine (1:1) to form acetyl derivatives. Nakamura and Pisano (37) derivatized the sample mixture (peptides, amino acids or amines) after spotting, by either dipping or spraying the plate with fluorescamine.

Note. When dipping or spraying is used, the reagent is dissolved in a solvent with which the sample is not miscible; otherwise runs or diffusion of the spots may occur which will affect resolution and quantitation.

Post development sample modification is mainly used for visualization and identification purposes. The sample after development is sprayed, dipped or placed in a gaseous atmosphere. Some spray reagents are specific. For example $Co(SCN)_2$ aqueous solution was found to be specific and can differentiate between a nitrite, a nitrate, and a nitrosamine by giving different colors; however, the reagent gives the same color for all nitrosamines tested (38).

In certain cases more than one reagent is needed. For example, nanogram amounts of adenine, guanine, uracil, cytosine and their alkylated bases, nucleotides and nucleosides were detected on TLC plate by placing the plate after development and drying in chlorine gas. When excess chlorine gas escaped the plate was sprayed with tolidine reagent to give colored spots (39). Kirchner (40) discussed reactions on the plate including oxidation, reduction, halogenation, hydrolysis, nitration and dehydration.

Many examples can be found in the literature (40-42) and need not be discussed here.

2. SOLVENT MODIFICATIONS:

a) Solvent Polarity: Solvent selection in TLC, as well as in HPLC (liquid adsorption and partition chromatography), is a function of the mixture to be separated and the adsorbent selected. Polar and nonpolar solvents are used with normal phases and polar solvents with reversed phases. The more viscous the solvent system, the slower the development, the less diffused the spots and the better the resolution. Changing the strength (polarity) of the solvent system affects not only the separation but also the R_f values. For example, in reversed phase TLC changing the percentage of water in the system may move a spot upward or downward depending on whether the water level is decreased or increased. In certain cases, changing from one alcohol to another affects the separation and R_f values. For example, changing from methanol to ethanol to n-propanol to isopropanol affected

the separation and the R_f values of alkylated adenines and uracils (43). The use of mixed mobile phases, although it has its advantages, as mentioned earlier, also has some disadvantages. It is not always easy to predict the R_f values as compared with those in pure solvent. R_f values may not be reproducible due to the evaporation of one of the components. Demixing (44) of the solvent components may take place which will affect the quality of the separation (45). Methods for the elimination of demixing have been discussed (45). Also, more diffused spots are obtained if less viscous solvents are used (46).

Solvent modifications in TLC are not new, they are probably as old as the technique itself. Whenever more than one component solvent system is used, it means that the researcher for one reason or another (separation, resolution, solubility, diffusion, visualization and identification) decided to add another solvent to his original one to aid in the chromatographic process. For example in the separation of amino acids and their derivatives if alcohols are replaced by low-polarity liquids in the solvent system which contains water, a solubilizer such as methanol pyridine or acetic acid is added to restore miscibility with water (46). Rasmussen (47) added fluoroscein to the solvent to aid in the UV detection of some acids on TLC plates. Aringer and Eneroth (1) and later Issaq et al (33) added HMDS to their solvent systems to achieve the separation of the oxidation products of cholesterol (Table 1).

b) Addition of Acids and Bases: Acids (46) or bases (43) are added to the solvent systems to prevent streaking and to produce more compact spots, when the separation of acids or bases is required, respectively. The addition of buffers and salts to control pH and ionic strength of water based phases is common in ion exchange chromatography (41).

c) Micellar Solutions: As was mentioned earlier, in soap TLC detergents are mixed with the adsorbent during slurry formation. In micellar

TABLE 1. Comparison of the Separation of Cholesterol and Six of its Oxidation Products, After Derivatization with TSIM, on Silica Gel, Silanized, and Reversed Phase Plates

Type of Plate	C_{18}	HMDS[1,2] Silanized		Silica Gel			Spray Reagent
Solvent: →	Heptane 48 HMDS 2			Ether 9**2 Cyclohex 1	Benzene 2[4] Heptane 8	Ether 1 Heptane 15	H_2SO_4 1 MeOH 1
Compound*	R_F x 100						Color
Cholesterol	93	66	76	75	77	84	Pink
7αOH	98	66	76	17	77	84	Reddish Brown
7βOH	62	46	49	26	51	46	Reddish Brown
7 keto	34	31	30	37	20	10	Dark Brown
α epoxide	54	41	45	40	47	36	Blueish
β epoxide	40	31	38	41	37	27	Blueish
3β,5α,6β triol	66	38	45	5	28	20	Blueish

*Cholesterols were derivatized with TSIM (Trimethylsilylimidazole)
**Underivatized

[1]HMDS = Hexamethyldisilazane

[2]Literature systems

[3]7-α and 7-β turned blue when the plate was sprayed with the reagent. Colors are shown under long UV and after heating in the oven at 110°C for approximately 5 minutes.

[4]0.25ml Acetonitrile was added to prevent streaking.

solvent systems surfactants are added to the aqueous mobile phase (48,49). A review of micelle forming surfactants was published (50). The principle of using micelle solutions is that partitioning does not occur to the bulk of the solvent but rather to highly selective species dissolved in the solvent. A simple micelle offers a variety of environments (50) from its organic core, to the more viscous polar region toward the edge of the hydrophobic core to the highly polar and charged (in the case of micelles formed from ionic surfactants) stern layer. The apparent polarity of a micelle mobile phase is altered by changing the concentration of surfactant in the solutions. The surfactants used were sodium dodecylsulfate, cetyltrimethylammonium bromide and Ipagal CO-710, a nonionic surfactant. The concentration of the surfactant in aqueous solutions ranged from 0.1-0.02M. They proved effective in the separation of pesticides, nucleosides and others on palyamide, and alumina but not on silica gel plates.

CONCLUSION

Modification in thin layer chromatography includes the adsorbent, the sample and the solvent system. Each of the above can be adjusted or manipulated to achieve the resolution of otherwise unseparable mixtures. Inclusion of chromophores in the layer or the solvent system may lead into visualization and subsequent identification of the separated compounds. Impregnation of the plate with metal salts and complexes, detergents and other materials will depend on the need and ingenuity of the researcher involved. Soap and ligand exchange chromatography seem to have a great potential in TLC separations.

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