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PREPARATION OF PLATES WITH A PERMANENT ADSORBENT LAYER AND THEIR APPLICATION IN THE ANALYTICAL THIN–LAYER CHROMATOGRAPHY OF LIPIDS

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SUMMARY

Experimental conditions for preparing the thin-layer chromatographic plates with a permanent adsorbent layer (PAL) have been established; the particles of this layer are firmly bound to each other and to the glass support by means of fused glass powder. To prepare the PAL plates, a mixture of Woelm silica gel and glass powder (1:3, w/w) of particle size 7 ± 1 μm was suspended in toluene and spread on the plates, yielding PALs of thickness 150–250 μm. The plates were heated in an electric furnace at 675° for 20 min. In the separation of neutral lipids, a PAL thus prepared is equal in efficiency and selectivity to silica gel layers prepared in the usual manner.

INTRODUCTION

At present, thin-layer chromatography (TLC) is a standard method for the qualitative separation of mixtures of organic compounds and the quantification of their individual components[1,2]. Although TLC has obvious advantages, it also has some serious drawbacks, in particular the fact that in most instances the worker himself is obliged to spread an adsorbent layer on plates for each chromatographic experiment. The uniform spreading of the adsorbent layer requires special equipment, certain technical skill and constant expenditure of time, and the chromatographic properties of the layers thus prepared are nevertheless difficult to standardize. In addition, the preparation of the plates in the laboratory in most instances involves the use of silica gel powder, which is harmful to health. Finally, many difficulties arise from the necessity of using binders while preparing the adsorbent layer. For example, if starch or poly(vinyl alcohol) is employed to fix the layer, the chromatographic zones cannot be rendered visible by means of strong mineral acids and heating[2,3]. If, on the other hand, gypsum is used as a binder, the separation of substances that form insoluble calcium salts, *e.g.*, acidic phospholipids, is impaired.[4]

Several workers have tried to reduce to the minimum these limitations of TLC. The first effort was made in 1968 by Taylor[5], who suggested a different approach to the fixation of the thin layer on a glass support. Employing a layer of highly porous

glass as an adsorbent, he fixed it on the plate by means of short-term heating. As a result, the powder and support fused to each other and the possibility arose of cleaning the plate with strong mineral acid mixtures after each chromatographic experiment so that repeated use would be feasible. The fixed layers thus obtained were used to separate standard dye mixture and also alkaloids. In 1972, Taylor's method was modified by Okumura and co-workers[6,7], who used silica gel mixed with powdered glass in definite proportions as the adsorbent. The mixture was spread on the plate, which was then subjected to heating. As a result, the silica gel particles became firmly bound to each other and to the surface of the plate by the fused glass powder.

Our attempt to prepare plates as decribed by Okumura et al.[7] failed because their description is incomplete. Therefore, we developed a method for the preparation of plates with a permanent adsorbent layer (PAL) that would be at least equal in its efficiency and selectivity in the separation of mixtures to the layers obtained in the usual manner. We examined the influence of the grade of silica gel, particle size of the glass powder, the weight ratio of the powder and silica gel in the mixture and also the heating conditions on the chromatographic parameters. Stahl's dyes, soybean seed oil and standard neutral lipids were used as test mixtures for fractionation. The results obtained indicate that the method developed can be used to prepare PAL plates that possess optimal efficiency and selectivity for the separation of lipids.

EXPERIMENTAL

Preparation of the mixture of silica gel and glass powder
        Grossly crushed window glass (3–4 kg) was ground in a 5-l barrel of a ball-mill (LE-107, Hungary) for 32 h. The powder was screened, the $\leqslant$ 195-mesh fraction of particle size 5–30 μm was collected and additionally fractionated from the aqueous suspension by sedimentation. For this purpose, the 195-mesh fraction (1.3 kg) was suspended in 6 l of water and allowed to stand for 1.5 h; the supernatant was decanted and these operations were repeated until it became clear. After the last decantation, the whole of the supernatant was allowed to stand for a further 2.5 h and the residue suspended in a 2-l beaker filled with water; the residue was washed until the supernatant became clear. Sedimented glass particles were dehydrated with acetone and the powder of particle size 7 $\pm$ 1 μm was screened with a 195-mesh sieve in order to remove lumps. Silica gel (Silica Gel Woelm TLC Adsorbent, Woelm, Eschwege, G.F.R.) and glass powder were mixed in a weight ratio of 1:3.

Preparation of PAL plates
        The apparatus used to prepare PAL plates was assembled as shown in Fig. 1. The "hot" end (1) of a chromel–alumel thermocouple was inserted into a muffle furnace (2) through a hole (3), while the „cold" end (6) was positioned at the bottom of a glass tube (8) inserted into a Dewar vessel (9) filled with ice–water (5) and covered with a plastic cap (7). The Dewar vessel was placed in a cylindrical support (4). The thermocouple was connected with the input of a potentiometer (10), used to record temperature changes in the furnace; a reading of 1 mV on the potentiometer scale corresponded to 25°. Plates of glass (10 $\times$ 20 $\times$ 0.2 cm) were placed in an apparatus for spreading[2]. The mixture of silica gel and glass powder (24 g) was suspended in 45 ml of purified toluene and a 350-μm thick suspension layer was spread by means
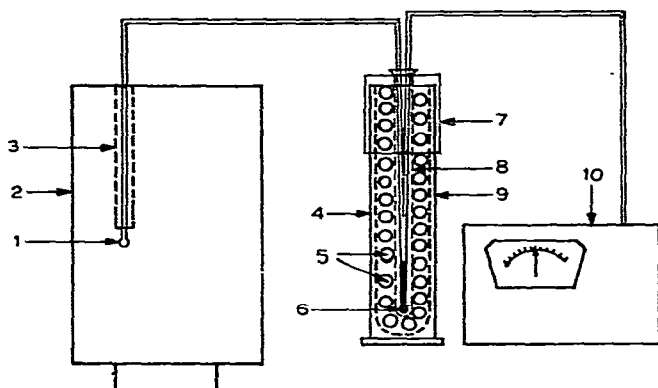
Fig. 1. Apparatus for the preparation of plates with a permanent adsorbent layer (PAL). 1 = "Hot" end of thermocouple; 2 = muffle furnace; 3 = hole for the thermocouple in the rear wall of the furnace; 4 = cylindrical support for Dewar vessel; 5 = pieces of ice; 6 = "cold" end of thermocouple; 7 = plastic cap, 8 = 45 × 2 cm glass tube; 9 = Dewar vessel; 10 = pH 340 potentiometer.

of a spreader[2]; the amount of the mixture mentioned above was sufficient to prepare five plates. After evaporation of toluene, the plate was placed on a graphite block (21 × 11 × 1 cm) lying on a stainless-steel support (Fig. 2,I), which was then inserted by means of a lever (Fig. 2,II) into a muffle furnace heated at 100–150°. The temperature was increased to 675°, the plate was left at this temperature for 20 min and then the furnace was gradually cooled to room temperature. Uniformity of the thickness of the PAL was controlled by means of a micrometer.

Immediately after preparation and also after each chromatographic experiment, the PAL plates were cleaned with concentrated sulphuric acid–nitric acid (9:1) at 100–120° for 30 min, then the plates were cooled, washed for 1 h with tap water and activated at 110° for 30 min.

Preparation of test mixture solutions for separation on PAL plates

A mixture of indophenol, Sudan Red G and Butter Yellow according to Stahl[8] (Test Mixture, Desaga, Heidelberg, G.F.R.) was used as a standard.
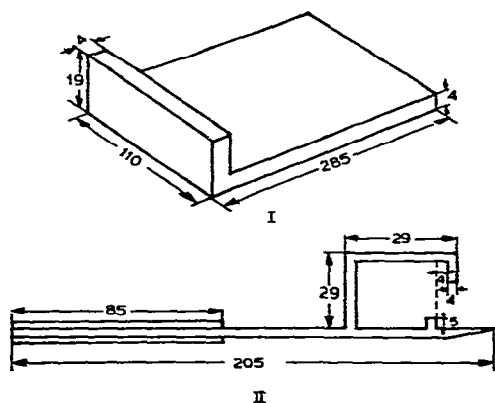

Fig. 2. Stainless-steel support and steel lever with a plastic handle. I = Support, general view; II = lever, side view. Dimensions are in millimetres.

Soybean seed oil was extracted as described previously[9] and dissolved in benzene to give a concentration of 100 mg/ml. To this solution, 0.01 % of butylated hydroxytoluene (BHT) was added.

Standard preparations of mixed monoglycerides of palmitic and stearic acids, mixed diglycerides of the same acids and also cholesteryl palmitate were used without further purification. Cholesterol was purified on an alumina column [activity II, benzene–acetone (1:1) used as eluent]. Triglycerides were extracted from soybean oil[9] and purified by the same procedure [n-hexane–benzene (3:2) used as eluent]. Free fatty acids were products of the saponification of soybean oil[10]; a proportion of these acids was converted into the methyl esters[9]. The purity of the preparations was checked by TLC on PAL plates with n-hexane–diethyl ether–acetic acid (80:20:1). In all instances the content of the major component in the preparations was more than 97 %. Standard neutral lipids were dissolved in benzene, the concentration of each class of lipid in this solution being 4 mg/ml.

Separation of substances on PAL plates

The separations were performed in 20.5 × 14-cm cylindrical chambers saturated with the vapour of the solvent to be used (saturation time 45 min) and covered with ground covers. To each solvent 0.01 % of BHT was added.

The standard dye mixture solution (2 μl) was applied on the layer by means of a Hamilton Microliter Syringe 10–12 mm above the lower edge of the plate; benzene was used as the solvent.

Soybean seed oil (100 μg) and a standard neutral lipid mixture (56 μg) were separated in n-hexane–diethyl ether–acetic acid (90:10:1 and 80:20:1). To render the lipid spots visible, the plates were treated with a 7% solution of molybdophosphoric acid in 45% aqueous isopropanol and heated at 120° for 5 min[2].

Standard lipids were also fractionated in a Wöelm silica gel layer without the glass admixture; the layers were spread on the plates by means of a spreader as described above.

Determination of chromatographic separation parameters

The relative retentions of the substances were expressed by R_M values[11], the selectivity of the separation by the number of chromatographic zones obtained after fractionation of a given mixture of substances and the separation efficiency by the number of theoretical plates (N) and HETP value (H)[12] for a given substance.

RESULTS AND DISCUSSION

Influence of the grade of silica gel on the chromatographic parameters of PAL plates

The chemical nature and the quality of the adsorbent are among the major factors that influence chromatographic separations[13]. In particular, the adsorption properties of silica gel depend largely upon the mode of its preparation[14]. Many grades of silica gel are available, manufactured by different firms with various degrees of purity, presence of binders, particle sizes, pore diameters, specific surface areas, inclusion of the fluorescent indicators, etc. Okumura et al.[7] did not specify the grade of silica gel they used, so it was essential to establish which grades were suitable for the preparation of the PAL plates to ensure the most efficient and selective separation of

TABLE I

INFLUENCE OF THE GRADE OF SILICA GEL ON THE RELATIVE RETENTION OF SUBSTANCES AND ON THE SELECTIVITY AND EFFICIENCY OF SEPARATION OF THE STANDARD DYE MIXTURE AND SOYBEAN OIL BY THIN-LAYER CHROMATOGRAPHY ON PAL

Silica gel grade	Particle size (μm) *	R_M		Number of chromato-graphic zones		N		H (mm)	
		TG** of soybean oil	Butter Yellow	Soybean oil	Dye mixture	TG** of soybean oil	Butter Yellow	TG** of soybean oil	Butter Yellow
Woelm	15 ± 3	0.05	0.21	12	3	83	812	0.80	0.07
Merck	15 ± 10	0.24	0.37	8	3	13	324	3.24	0.14
Chemapol	20 ± 14	0.50	0.43	8	3	4	282	5.01	0.15

* Measured in the present work; $\bar{x} \pm S$ values are given, where $\bar{x}$ is the arithmetic mean of the particle size for 30 measurements and S is the absolute standard deviation[10].
** TG = triglycerides.

lipids. In our work, several common grades of silica gel were investigated, namely Woelm silica gel (see Experimental), LS-5/40 silica gel, (Chemapol, Prague, Czechoslovakia) and Kieselgel G (Merck, Darmstadt, G.F.R.); the last type was washed with hydrochloric acid until free from the binder (calcium sulphate) (these silica gels will be referred to below as Wöelm, Chemapol and Merck, respectively). For the preparation of the PAL, the glass fraction of particle size $7 \pm 1 \mu m$ was used (see below).

It can be seen from Table I that the plates obtained from the various silica gels have different chromatographic properties, the lowest relative retention (R_M) and the highest selectivity and efficiency of separation of the standard mixtures being shown by PAL prepared from the Wöelm silica gel. It can be that in this instance the result obtained is due to the differences in the particle sizes, which is supported by the well known property in "conventional" TLC that the smaller the particles used for the preparation of the adsorbent layer and the narrower the range of their size variations, the higher is the efficiency of separation of compound mixtures[12]. It can be seen from Table I that of the grades of silica gel examined the Woelm silica gel demonstrated these properties most effectively; it is not unlikely that its particle size did not change upon heating, as its melting point was not reached. We used only this grade of silica gel in subsequent work.

Influence of the original size of the glass powder particles and of the glass powder to silica gel weight ratio in the layer on the chromatographic parameters of PAL plates

Okumura and co-workers[6,7] claimed that, in order to prepare the silica gel layer fixed on the plate by means of fused glass powder, the particles of glass and the adsorbent must have similar particle sizes, but no recommendation was given of the exact size of these particles. To check this claim and to determine the optimal particle size of the glass for the preparation of PAL, the "original" $\leqslant$ 195-mesh (11 ± 10-μm) glass fraction was used in our preliminary experiments. However, PAL prepared from the mixture of this fraction and Wöelm silica gel (15 ± 3 μm) did not give a sufficiently good separation of the minor components of soybean seed oil (Table II). We considered that the chromatographic properties of the layer might be improved by a further decrease in the particle size of the glass and a simultaneous narrowing of

TABLE II

INFLUENCE OF THE GLASS PARTICLE SIZE ON RELATIVE RETENTIONS AND ON THE SELECTIVITY AND EFFICIENCY OF SEPARATION OF THE STANDARD DYE MIXTURE AND SOYBEAN OIL BY THIN-LAYER CHROMATOGRAPHY ON PAL

Glass powder fractions in PAL*	Particle size (μm)	R_M		Number of chromatographic zones		N		H (mm)	
		TG of soybean oil	Butter Yellow	Soybean oil	Dye mixture	TG of soybean oil	Butter Yellow	TG of soybean oil	Butter Yellow
"Original" (≤195 mesh)	11 ± 10	0.20	−0.01	10	3	23	1024	1.96	0.08
1	21 ± 11	−0.01	−0.05	9	3	35	717	1.78	0.12
2	7 ± 1	0.05	0.08	12	3	83	1837	0.80	0.04
3	5 ± 1	−0.08	0.30	5	3	39	218	1.81	0.22

* Glass to silica gel weight ratio in PAL, G/SG = 3:1.

the range of their sizes. In fact, when fraction 2 (7 ± 1 μm) was used, the selectivity and efficiency of separation of the components of both soybean oil and the standard dye mixture were considerably improved compared with the original fraction. This size seems to be optimal, since an increase or decrease from this value adversely affected the chromatographic parameters of PAL (Table II, fractions 1 and 3). Therefore, only 7 ± 1-μm glass particles were used in subsequent experiments.

The results reported by Okumura and Kadono[6] suggest that the R_F values of steroids depend on the weight ratio of glass to silica gel (G/SG) in the layer; according to these authors, the optimal range of this ratio is from 2:1 to 5:1. To investigate the possible influence of this factor on the relative retention (R_M) and separation efficiency, we fractionated standard mixtures of dyes and neutral lipids in PAL having G/SG ratios of 1:1, 3:1 and 5:1.

Table III shows that in most instances the R_M value decreases and the separation efficiency increases with an increase in this ratio. At the same time, the maximal amount of lipids that can be separated without a loss of linearity of the adsorption isotherm decreases because, with a decrease in the silica gel content in the PAL, the linear capacity[12] of the layer is reduced. Therefore, the use of layers with G/SG ≥ 5:1 would lead to a considerable decrease in the maximal weight of the sample that can be separated. Apparently, the linear capacity of the layer can be enhanced if the silica gel content in the PAL is increased; however, at G/SG < 3:1 the content of glass in the layer becomes insufficient to ensure the necessary mechanical durability of the PAL. Consequently, to prepare PAL plates with a maximal linear capacity, adequate durability and acceptable chromatographic efficiency, the G/SG ratio of 3:1 must be used. Our results for the optimal particle size of the glass and the G/SG ratio are not in conflict with those of Okumura and co-workers[6,7], but they give much more precise information.

After being spread on the surface of a glass plate, the thin layer of silica gel-glass mixture is highly porous, which enhances rapid and efficient separations. To prevent deterioration of porosity during heating, the temperature at all points in the layer must be the same at all times during both heating and cooling of the plate. The

TABLE III

INFLUENCE OF THE GLASS TO SILICA GEL WEIGHT RATIO IN PAL ON RELATIVE RETENTIONS AND THE EFFICIENCY OF SEPARATION OF THE STANDARD DYE MIXTURE AND NEUTRAL LIPIDS BY THIN-LAYER CHROMATOGRAPHY ON PAL

Substance separated*	Glass to silica gel weight ratio								
	1:1			3:1			5:1		
	R_M	N	H (mm)	R_M	N	H (mm)	R_M	N	H (mm)
Indophenol	1.65	36	0.08	·1.38	44	0.11	1.24	39	0.18
Sudan Red G	1.06	144	0.08	0.69	467	0.06	0.62	334	0.10
Butter Yellow	0.40	225	0.20	0.08	1837	0.04	−0.10	1708	0.05
MG	1.76	16	0.13	1.54	36	0.08	1.59	16	0.19
DG	0.79	196	0.11	0.46	747	0.10	0.48	747	0.05
Cholesterol	0.58	272	0.12	0.37	455	0.11	0.36	494	0.10
FFA	0.13	625	0.12	−0.17	1283	0.08	−0.23	1044	0.10
TG	−0.29	743	0.15	−0.45	1681	0.07	−0.55	2329	0.06
FAME	−0.54	1600	0.08	−0.64	3003	0.05	−0.71	4153	0.04
SE	−1.10	2844	0.07	−1.06	4869	0.03	−1.14	6561	0.03

* Neutral lipids: MG = monoglycerides; DG = diglycerides; FFA = free fatty acids; TG = triglycerides; FAME = fatty acid methyl esters; SE = sterol esters.

optimal temperature and duration of heating to ensure the preparation of a highly porous and sufficiently strong PAL should be determined experimentally. They depend on the melting point of the glass used and on its content in the layer; in our experiments, the plate with the applied layer (G/SG = 3:1) was heated to 675° and left at this temperature for 20 min.

Separation of neutral lipids on PAL plates

Fig. 3 shows the chromatogram öf soybean seed oil (I and II) and a standard mixture of neutral lipids (III and IV) obtained on PAL (G/SG = 3:1) and on a thin layer of Wöelm silica gel without the admixture of glass; n-hexane–diethyl ether–acetic acid (80:20:1) was used as the developing solvent. It can be seen that on PAL both mixtures are separated fairly well, while no adequate separation of minor components of the oil was achieved on the silica gel layer obtained in the usual manner, and during fractionation of the standard mixture of neutral lipids on this layer diglycerides were not separated from cholesterol.

Table IV gives the values of R_F, R_M, N and H during the separation of neutral lipids on PAL and on a "loose" Woelm silica gel layer. It can be seen that PAL gives a higher efficiency of lipid fractionation than the silica gel layer obtained in the usual manner. It is possible that one of the reasons for this difference is that, provided the same solvent is employed, lipids have a lower relative retention (R_M) on PAL than on a "loose" silica gel layer. The same reasoning applies to the different selectivities of the separation of diglycerides and cholesterol on these layers.

A comparison of our results with those for lipid separations using conventional TLC systems reported by other workers (see Table V and also Fig. 130 in ref. 18) leads to the general conclusion that, as regards the selectivity and efficiency of separation of both standard and natural mixtures of neutral lipids, PAL is comparable to
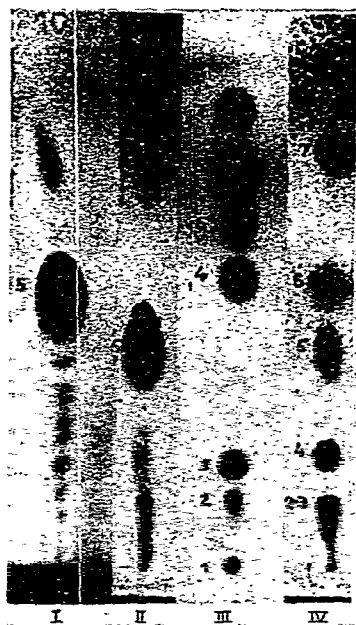
Fig. 3. Chromatograms of soybean seed oil and a standard mixture of neutral lipids. I and II = soybean oil; III and IV = standard mixture of neutral lipids. I and III = PAL, G/SG = 3:1; II and IV = Woelm silica gel layer containing no glass. Spots: 1 = monoglycerides; 2 = diglycerides; 3 = cholesterol; 4 = free fatty acids; 5 = triglycerides; 6 = fatty acid methyl esters; 7 = sterol esters. Components of soybean oil other than triglycerides were not identified.

TABLE IV

INFLUENCE OF THE PROPERTIES OF THE SILICA GEL ADSORBENT LAYER ON RELATIVE RETENTIONS AND THE EFFICIENCY OF SEPARATION OF THE STANDARD MIXTURE OF NEUTRAL LIPIDS BY THIN-LAYER CHROMATOGRAPHY

Neutral lipid	PAL (G/SG = 3:1)				Woelm silica gel layer without glass admixture**			
	hR_F*	R_M	N	H (mm)	hR_F*	R_M	N	H (mm)
MG	3	1.54	36	0.08	—	—	—	—
DG	26	0.46	747	0.10	—	—	—	—
Cholesterol	30	0.37	455	0.11	10	0.96	455	0.04
FFA	60	−0.17	1283	0.08	18	0.65	314	0.10
TG	74	−0.45	1681	0.07	37	0.11	476	0.13
FAME	81	−0.64	3003	0.05	48	0.04	868	0.09
SE	92	−1.06	4869	0.03	72	−0.41	3782	0.03

* $hR_F = R_F \times 100$ (ref. 15).

** — indicates that hR_F, R_M, N and H were not measured because DG were not separated from cholesterol and MG were on the origin of the chromatogram (see Fig. 3, IV).

silica gel layers that contain no glass. In the work of Itoh et al.[16], plates prepared according to Okumura were used for lipid separation. The separation pattern of the standard mixture of neutral lipids obtained in our work agrees well with that of Itoh et al. (see Table V).

TABLE V

hR_F VALUES OF NEUTRAL LIPIDS AFTER SEPARATION ON PAL AND SILICA GEL THIN LAYER WITHOUT GLASS ADMIXTURE

Solvent systems: n-hexane–diethyl ether–acetic acid (80:20:1) (this work); light petroleum–diethyl ether–acetic acid [90:10:1, refs. 16 (a) and 19, and 80:20:1, ref. 16 (b)]; benzene–diethyl ether–ethyl acetate-acetic acid (80:10:10:0.2) (ref. 19); n-heptane–diisopropyl ether–acetic acid (60:40:4) (ref. 20). In ref. 16, silica gel plates prepared according to Okumura et al.[7] were employed and in refs. 17–20 the silica gel layer without glass admixture was used.

Neutral lipid	hR_F						
	This work	Data from the literature					
		Ref. 16(a)	Ref. 16(b)	Ref. 17	Ref. 18	Ref. 19	Ref. 20
MG	3	—	15	8	2	0	5
1,2-DG	26	7	61	59	15	8	25
1,3-DG	26	11	68	72	21	8	40
Cholesterol	30	15	57	48	19	10	30
FFA	60	43	82	20	39	18	50
TG	74	52	89	80	60	30–40	82
FAME	81	75	—	—	77	65	—
SE	92	90	—	100	94	90	90

Hence the application of PAL plates makes it possible to overcome the limitations of TLC mentioned above. Moreover, these plates have certain advantages over the usual plates, the major one being that the same layer can be used many times for the analysis of mixtures without changes in its properties. It is clear that the selection of optimal conditions for the separation of a given mixture can thus be considerably accelerated. Moreover, application of PAL plates facilitates the impregnation of the adsorbent with different substances, because it can be immersed in any liquid except hydrofluoric acid and concentrated alkali solutions without any damage to the layer. Impregnation has been employed for the modification of layers prior to separation, and also for rendering visible the chromatographic zones formed[2,21]; up to now, spraying has been used for this purpose, but it does not ensure an even distribution of the substance on the surface of the adsorbent and can contaminate the laboratory with corrosive compounds or due stuffs. Finally, the application of PAL plates will make it possible to facilitate and to accelerate considerably the standardization of a densitometric determination of separated substances because, while performing a series of experiments, chromatographic analysis of the mixture can be effected repeatedly with the same PAL.

Various firms (Chemapol, Merck, Applied Science Laboratories and others) produce plates with a ready-made silica gel layer under such trade-names as Silufol, PSC-Fertigplatten and Prekotes. These plates are used by many workers because of the superior uniformity of the layer thickness compared with those of plates prepared in the laboratory. However, it should be stressed that the pre-coated plates suffer from some of the drawbacks mentioned above. In both instances the plates can be used only once; precautions should be taken while using the plates in order to prevent damage of the layer, and commercially available TLC plates on glass supports are expensive and cumbersome to handle. When a metal foil serves as the support, the plates cannot be used for densitometry in transmitted light. The difficulties that arise owing to the

presence of organic or mineral binders in the layers, and also while performing the impregnation of such layers with certain substances, have already been noted. It seems that in the future, plates with a ready-made layer will be replaced by PAL plates, which are free from the above limitations.

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