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# PROBLEMS AND APPLICATIONS OF REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

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## SUMMARY

The various technical problems encountered in the use of chemically bonded silica in thin-layer chromatography (TLC) are discussed. While the use of alkyl grafted silica offers all the advantages of reversed-phase systems for use in TLC, some practical difficulties arise because polar solvents do not completely wet the modified surface. Water–alcohol mixtures with water concentrations above 30% migrate very slowly within the porous layer, resulting in prohibitively long analysis times. Migration over distances exceeding a few centimetres becomes erratic and poorly reproducible. Other problems are encountered with sampling and detection.

The plates are very easy to use and permit extremely good separations that were previously very difficult to achieve, especially for polar compounds. Some examples are given. The preparation of samples is especially easy as aqueous solutions need simply be diluted with methanol.

## INTRODUCTION

The practical applications of reversed-phase (RP) liquid chromatography are becoming more numerous and more important in column liquid chromatography than those of normal chromatography, because the development of chemically bonded phases offers a set of stationary phases that are easier to use and more reliable than physically coated supports. Karger and Giese[1] showed recently that chemically bonded phases are well suited for the analysis of polar and complex substances because they are selective, the retention can be adjusted rapidly by appropriate change of the composition of the mobile phase, these changes can be made quickly, they

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are tolerant to experimental abuse and are a good tool for the characterization of substances.

Analysts have been willing to use the same material in thin-layer chromatography (TLC) and, as an example, we have previously described the separation of alkylpyridines on RP-18 [2,3]. Unfortunately, the plates prepared without a binding agent were very fragile, turning into a cloud of dust in a light draught of air, and few attempts have been made to carry out TLC analyses with bonded phases[3–5].

This difficulty has been overcome and pre-coated plates made with 5-$\mu$m silica particles chemically bonded with an ethyl, an *n*-octyl or an *n*-octadecyl group are now commercially available.

The use of these plates is easy provided that a convenient solution to a few specific problems discussed below is obtained. The main advantage over plates coated with plain silica particles is the need for much simpler solvent mixtures, the better reproducibility and the lack of influence of solvent demixing, at least at water concentrations below about 30%. The spots of individual components are much narrower and more regular and the separation of complex mixtures is considerably easier. These plates are also much easier to prepare and handle than those made with silicones or paraffin-impregnated silica, in which case the silica surface remains polar and its activity is difficult to control.

## EXPERIMENTAL

RP-2, RP-8 and RP-18 TLC plates were obtained from Merck (Darmstadt, G.F.R.). Some KC-18 plates from Whatman (Clifton, N.J., U.S.A.) were also used.

Some plates have been prepared successfully by a standard coating procedure using *n*-octadecane as a binding agent. Spreading is effected by using a suspension of RP-18 (5-$\mu$m particles) in a dilute solution of *n*-octadecane in *n*-pentane. Because of the low viscosity of this solvent, the slurry tends to filter rapidly out of the spreading device, so the narrowest slit is used and the thickness of the layer is only 0.1 mm. The solvent dries very rapidly. These plates have properties very similar to those of commercial plates.

## RESULTS AND DISCUSSION

### *General behaviour of the plates*

In reversed-phase chromatography, the mobile phase is usually a mixture of water and a polar solvent such as methanol, acetonitrile, acetone or an ether. Such mixtures do not wet completely silica chemically bonded to alkyl chains, and therefore some problems arise in the use of these plates.

*Sample application.* The solute must dissolve in a solvent with an eluotropic strength lower than that of the solvent used for development, in order to prevent excessive spreading of the original spot. The plates are hydrophobic, however, and a drop of a water-rich solution does not penetrate into the layer but rolls on the plate. In most instances methanol, which is quickly evaporated and wets the layer properly, is convenient for substances capable of hydrogen bonding. For less polar compounds, including aromatic hydrocarbons and carotenoids, methylene chloride and acetone are convenient.

We found sampling to be easier on the Whatman plates as they are wetted by organic solutions containing up to 50% of water.

As the layer is made with 7-$\mu$m particles, it is potentially capable of a high efficiency[6–9], provided that sample application results in narrow spots. Accordingly, very narrow tubes should be used and the amount of sample should not exceed 100 ng in favourable cases.

*Flow-rate of mobile phase.* In TLC this depends entirely on the choice of the characteristics of the thin layer and the solvent used. It is well known[9–11] that the migration distance, $z$, of the solvent front above the solvent level in the tank is related to time according to the following equations:

$$z^2 = kt \tag{1}$$

$$k = 2k_0 d_p \cdot \frac{\gamma}{\eta} \cdot \cos\theta \tag{2}$$

where $d_p$ is the particle diameter, $\gamma$ and $\eta$ the surface tension and viscosity of the solvent, respectively, $\theta$ the wetting angle of the adsorbent surface by the solvent and $k_0$ a parameter depending on the bed permeability, the pore distribution outside (around) the particles and the relationship between solvent front and bulk velocities[12]. In conventional adsorption TLC, $\cos\theta \approx 1$ with all solvents used. It is not the same with *n*-alkyl-grafted silica and $\cos\theta$ drops rapidly with increasing water content of the solvent[13]. At moderate water contents this adds to the effect of increasing viscosity of the solution. At higher water contents it offsets the effects of the decrease in viscosity and the increase in surface tension of the solution, at least as long as $\theta$ is more than 90°, as it is obviously impossible to develop a plate when $\theta$ is less than 90°, and in practice this becomes very difficult when $\theta$ becomes smaller than 130°.

Fig. 1 shows that the development time increases dramatically with increasing water content of acetonitrile and methanol solutions. Similar results were obtained with other alcohols, acetone and ethers. A 4-cm rise using methanol–water (60:40) on an RP-18 plate takes 90 min, which is prohibitively long. This phenomenon will be discussed in detail elsewhere[14] but there does not seem to be any means of increasing the development rate even if it is possible to use water-rich mixtures. In practice, development times exceeding about 20 min are too long so water concentrations greater than 20% in methanol and 40% in acetonitrile should be avoided.

*Reproducibility of retention data.* Silica readily adsorbs water and changes considerably in activity upon adsorption of small amounts of water. Accordingly, silica plates must be handled with caution so as to avoid exposure to moisture and be carefully dried immediately before use. Failure to do so results in erratic results. This problem does not exist with chemically bonded silica. We did not pay any attention to the time of exposure of the plate to ambient air, but nevertheless always achieved very reproducible $R_F$ values (relative standard deviation about 2–3%). During development with water–alcohol mixtures, however, the plate must be protected by a spacer in order to prevent adsorption of the alcohol vapour.

Reversed-phase TLC plates can be re-used after careful washing and drying, unless the layer is mechanically damaged. No noticeable decrease in efficiency was observed on plates that had been used several times.

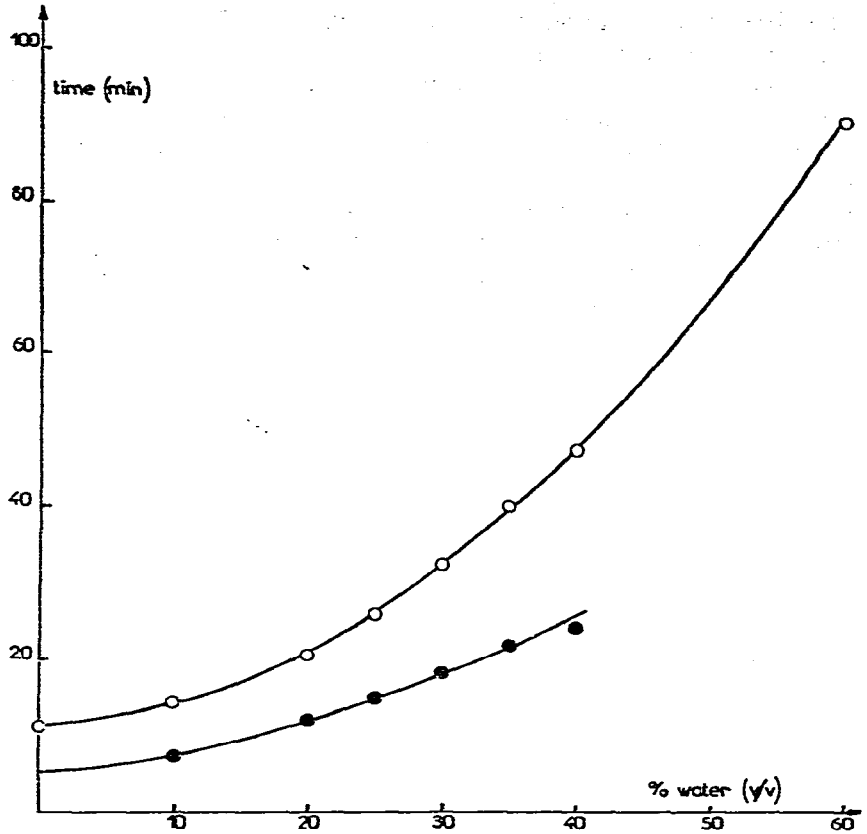



Fig. 1. Time required for a 4-cm development distance on an RP-18 plate using organic solvent–water mixtures as a function of the water content (volume-%). ●, Acetonitrile; ○, methanol.

*Detection problems.* Conventional charring procedures cannot be used because the organic material content of the plates is too high.

Commercial Whatman or Merck plates are fluorescent under UV illumination, the colours being yellow for Whatman and grey-blue for Merck plates. Spraying with a suitable indicator is necessary when a sample is not revealed under UV light. Spraying is possible, however, only with solutions in methanol or acetone or with Freon-type sprays; with water or water-rich solutions the plate is not wetted. This restricts drastically the number of reagents that can be used for detection. Furthermore, the spot shapes produced in this way are usually not convenient for quantitative work. Clearly this field will have to be carefully studied in the immediate future in order to provide specific reagents.

Classical detection is effected with spectrophotodensitometers. Working with Merck plates we have been unable to use light transmission with the UV lamp for quantitation, as is possible with silica HPTLC plates. The background absorption by the plate is very high and the intensity of the transmitted light does not permit accurate measurements. Probably the binder has a marked UV absorption, which prevents the use of the transmission mode. The reflection mode is well suited, on the

other hand, and reproducible results are obtained. It is known, however, that combined measurements of reflected and transmission light give better signal-to-noise ratios[15]. This mode is not possible with RP plates and the sensitivity at present is poorer than that with conventional plates.

*Selectivity of chemically bonded silica*

This study deals as much with correlation between the results obtained by TLC and column liquid chromatography as with selectivity itself, as this problem has already been largely studied in column chromatography. Retention data are correlated[16] according to the following equations:

$$R'_M = \log k' \tag{3}$$

$$R'_M = \log\left(\frac{1}{\xi R_F} - 1\right) \tag{4}$$

where $k'$ is the column capacity factor and $R_F$ the retention factor in TLC; $\xi$ takes into account the fact that the operating conditions are different in the two techniques[17]. With reversed phases, $\xi$ was very close to unity.

*Nature of stationary phase.* Ethyl, *n*-octyl and *n*-octadecyl groups are currently available. In column chromatography, $R'_M$ increases linearly with the number of carbon atoms in a homologous series, beyond the first few members of the series[18]. The same result is observed in TLC. Data obtained for alkylphenones, from valerophenone to tetradecylphenone, using RP-18 (Merck) particles and methanol as the eluent in column chromatography and TLC correlate very well. The following equations were obtained:

$$\text{RP-2:}\quad R'_M = -1.08 + 0.0309\,n \tag{5a}$$

$$\text{RP-8:}\quad R'_M = -0.86 + 0.0558\,n \tag{5b}$$

$$\text{RP-18:}\quad R'_M = -0.80 + 0.0711\,n \tag{5c}$$

where $n$ is the number of carbon atoms or increments in the alkyl chain. The correlation coefficient is greater than 0.995 in all three cases.

The selectivities for homologues, given by the ratio $\alpha = [k'(n+1)/k'(n)]$ (*cf.*, eqn. 3), are 1.074 (RP-2), 1.14 (RP-8) and 1.18 (RP-18), indicating that the selectivity increases with increasing length of the grafted alkyl group. This result is in agreement with high-performance liquid chromatographic (HPLC) results[19,20].

*Effect of mobile phase.* Retention and selectivity depend on both the water content of the solvent and the nature of the organic modifier used. In reversed-phase liquid chromatography water is the least eluotropic solvent. Increasing the water content of the solvent mixture is an easy means of adjusting the retention.

We have determined in the classical, vertical TLC mode the variation of the retention data of some compounds with water content. We observed, as expected, linear plots of $R'_M$ for a large number of solutes (alcohols, phenols, esters and more complex compounds such as testosterone, caffeine, progesterone and fluoranthene) *versus* the concentration of solvent in a water–organic solvent mixture. The slopes of the lines corresponding to the different solutes are very close. A 10% increase in the

water content yields a *ca.* 1.85 fold increase in retention ($R'_M$ increases by 0.27). For very polar solutes the $R'_M$ values increase more slowly with increasing water content. This result is in marked disagreement with the findings of Biagi *et al.*[21], who obtained a marked deviation from linearity at low acetone concentrations, a substantial scatter of results and a significant increase in the slope of the linear portion of the graph with increasing retention. Their results can easily be understood, however, as they used silicone oil-impregnated silica, which is a very poor system, combining all the inconveniences of silica and of a reversed-phase system with little of the advantages[1].

Retention data ($R'_M$ values) for pyrene are plotted in Fig. 2 *versus* the molar fraction of water, using three different solvents. For other solutes, such as caffeine, testosterone and progesterone, lines parallel to those corresponding to pyrene were obtained, but are not reported for the sake of clarity. Pyrene was chosen because the $R_F$ values were between 0.1 and 0.90 and $R'_M$ between +0.95 and −0.95. Outside that range, the experimental results were inaccurate.

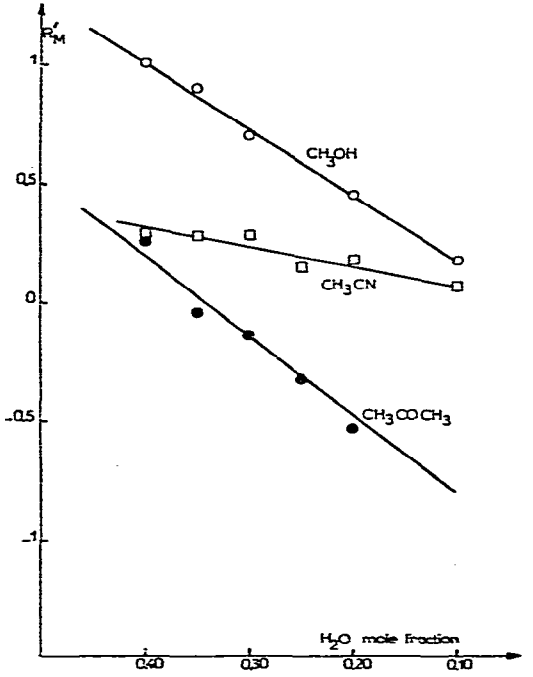



Fig. 2. Variation of the retention of pyrene with the composition of a water–organic modifier mixture. ○, Methanol; □, acetonitrile; ●, acetone. Plate: Merck RP-18.

It can be seen that the plots for methanol, acetonitrile and acetone have different slopes. The change in acetonitrile concentration has a weak effect on the retention, whereas a change in acetone concentration has a greater effect than the same change in methanol concentration. Thus, Fig. 2 demonstrates that a change in the solvent composition may modify the retention much more than a change in the water content and permits a quicker analysis. These variations are useful for adjusting

the retention of a solute to the desired value in order to optimize a separation.

Usually organic acids are analysed in conventional adsorption TLC using mixtures containing acetic acid. The spots exhibit strong tailing owing to adsorption by silica and solvent demixing frequently occurs, with the characteristic appearance of a second front line. With bonded phases similar solvents are used, but the TLC spots are circular with no detectable tailing; control of the pH of the mobile phase is, of course, critical for achieving reproducible retention data. The best analytical conditions are achieved when the pH of the mobile phase is markedly lower than the $pK_a$ of the acid.

The variation of $R'_M$ for a series of substituted benzoic acids with the concentration of methanol in a methanol–water mixture is linear[18]. In these experiments the acid concentration in water was kept constant at 0.1 *M*. The retention and the slope of the straight line of $R'_M$ *versus* methanol concentration increases when solutions of stronger acids are used.

It has been pointed out[1] that bonded phases may be slowly attacked when solutions of low pH are used. While this may limit the lifetime of HPLC columns, there is no problem with bonded phase TLC plates which are used for one run or a few runs only, with an amount of solution which does not exceed one bed volume each time.

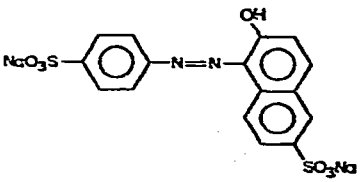
*Application to some analytical problems*

These separations are given as an example of what can be achieved easily and rapidly with the new reversed-phase TLC plates. They have not been optimized and could certainly be markedly improved. Only photometric records of spot profiles are given as we are of the opinion that drawings of spots on a plate are meaningless[8].

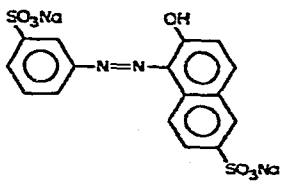
*Separation of closely related isomers.* The separation of chlorophylls has been the topic of many papers[22]. It is difficult because of the lability of the compounds and because problems arise when recovery of these substances is needed. Reversed-phase chromatography was especially inconvenient and messy. Sherma and Latta[23] recently published a separation of chlorophyll *a* and *b* by reversed-phase TLC using $C_{18}$ silica, and Rebeiz and Castelfrancs[24] performed a reversed-phase HPLC separation with a ternary solvent.

Using the same solvent (methanol–tetrahydrofuran–water, 75:20:5), we were able to separate chlorophyll *a* and *b*, and also pheophytin *a* and *b*, which were not analysed by Sherma and Latta[23]. Chromatograms obtained on RP-8 and RP-18 are shown in Fig. 3. The separation appears better on RP-8 because the $R_F$ value corresponds to the best efficiency zone of the plate[9]. The use of a solvent with a greater eluotropic strength than that chosen by Sherma and Latta[23] permits the analysis of pheophytin *a* and *b*.

*Separation of GGN Yellow (I, known as E 111 dye) and Yellow S (II, known as E 110 dye).* These are very similar compounds, as shown in the formulae.




I

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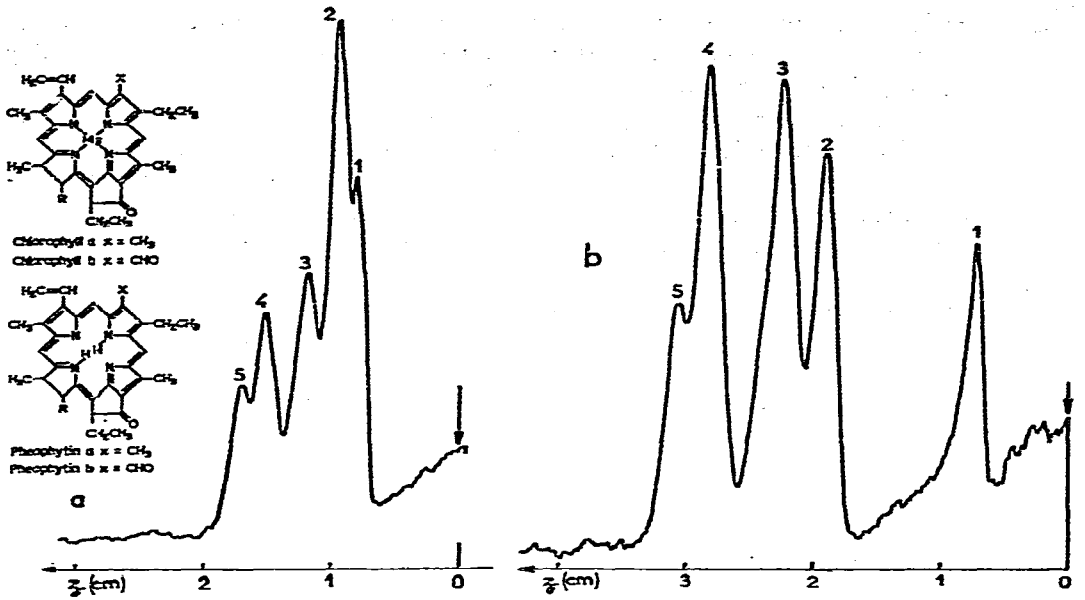


Fig. 3. Separation of plant pigments. Solvent: methanol–tetrahydrofuran–water (75:20:5). (a) RP-18 plate; (b) RP-8 plate. Recording with Zeiss PMQ II, reflection mode, $\lambda = 630$ nm. Plate travel, 30 mm/min. Chart paper speed, 120 mm/min. Peaks: 1 = unknown; 2 = pheophytin *a*; 3 = pheophytin *b*; 4 = chlorophyll *a*; 5 = chlorophyll *b*.

E 111 (I) is forbidden in food additives, but E 110 (II) is allowed. Their separation is very difficult and is usually carried out on the degradation products, sulphanilic and metanilic acid, respectively. These two isomers were separated on an RP-18 plate (Fig. 4) using as the mobile phase methanol–0.1 *M* acetic acid (30:70). The $R_F$ values are 0.49 for E 110 and 0.28 for E 111. The possibility of spotting many solutions on one plate is very convenient for the routine analysis of numerous samples.

*Separation of sugars.* Heftmann[25] lists about 45 references to chromatographic separations of sugars. A large number of systems have been used, but apparently none is completely satisfactory. Cellulose[26] and silica[27] are mainly used in current practice. Certainly there is a need for good reversed-phase liquid chromatographic

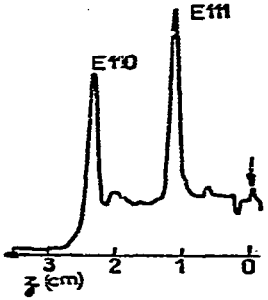


Fig. 4. Separation of yellow dyes E 111 and E 110 on an RP-18 plate. Development distance: 5 cm. Mobile phase: methanol–0.1 *M* acetic acid (40:60). Detection: reflection mode.

systems. As an example, ribose, arabinose and xylose are easily separated on an RP-18 plate with water–acetonitrile (12:88) as the mobile phase, the $R_F$ values being 0.83, 0.75 and 0.53, respectively. The separation achieved is much better than that on LiChrosorb $NH_2$.

The detection of sugars is very difficult. It is carried out using a solution of vanillin in methanol as the spray reagent. The detection and retention data are accurate enough only for identification purposes and a quantitative determination is not possible.

*Separation of some dyes.* E 102, tartrazine [sodium salt of 3-carboxy-5-hydroxy-1-(*p*-sulphophenyl)-4-(*p*-sulphophenylazo)pyrazole] is a green dye long used in mint syrups and candies, which is expected to be forbidden in the near future. It can be separated easily from the allowed E 131 (Blue Patent V, calcium salt of disulphonic *m*-hydroxytetraethyldiaminobiphenylcarbinol), using methanol–0.1 *M* acetic acid (40:60) (pH 3.5) on RP-18 plates. The $R_F$ values were 0.90 for E 102 and 0.75 for E 131.

Separations of these dyes on cellulose[28] and silica[29] have been published. In these instances the dye must be extracted first from the syrup with an organic solvent. If a reversed-phase system is used, a sample of the product for analysis of the dyes need only be diluted with methanol, which is much simpler.

*Separation of some acid mixtures.* Oleic and linoleic acids are well resolved on an RP-18 plate using as the solvent system methanol–0.1 *M* acetic acid (70:30). This allowed the direct chromatography of fatty acids extracted from sunflower oil. It was seen on the plate that the saturated $C_{16}$ (palmitic) and $C_{18}$ (stearic) acids are only minor components. They were detected by spraying with a molybdophosphate solution.

The use of a reversed-phase system is again a notable improvement over the conventional TLC analysis with silica, which requires the conversion of the acids into methyl or benzyl esters prior to the separation[30]. In the present method the sample for analysis need only be diluted with methanol.

This method also permits a very rapid detection of erucic acid.

Sorbic and benzoic acids are usually separated on adsorption plates with chloroform–water (65:35) (pH 4)[31]. Although this separation is good, we prefer the use of ion-pair chromatography. This now well known technique is based on the formation of a zwitterion according to the equilibrium

$$\text{Solute}^{\pm} + \text{counter ion}^{\pm} \leftrightarrows [\text{solute}^{\pm}\text{–counter ion}^{\pm}]$$

On an RP-2 plate with tetrabutylammonium as the counter ion, the resolution is excellent and allows a precise quantitative analysis. Ion-pair chromatography on reversed-phase plates will be very useful either for performing simple analyses or as a convenient means of selecting the best system in HPLC. Two methods are possible, either to pre-wet the plate with the counter-ion solution, or to perform the analysis directly on the reversed-phase plate. The former method gives better results.

*Separation of substituted benzoic acids.* Substituted benzoic acids are very polar and difficult to separate by conventional TLC. Application of the results discussed above makes it possible to enhance the selectivity and increase the resolution. We used methanol–0.1 *M* acetic acid mixtures of various compositions as the mobile

TABLE I

$R_F$ VALUES OF SUBSTITUTED BENZOIC ACIDS

| *Methanol concentration (%)* | $R_F$ *values* | | | | |
|---|---|---|---|---|---|
| | *Benzoic acid* | *Vanillic acid* | *p-Hydroxybenzoic acid* | *2-Bromobenzoic acid* | *Anisic acid* |
| 20 | 0.095 | 0.22 | 0.33 | 0.04 | |
| 30 | 0.17 | 0.34 | 0.44 | 0.10 | 0.07 |
| 40 | 0.22 | 0.48 | 0.58 | 0.16 | 0.14 |
| 60 | 0.52 | 0.70 | 0.79 | 0.47 | 0.45 |
| 70 | 0.66 | 0.805 | 0.84 | 0.65 | 0.58 |
| 80 | 0.805 | 0.91 | 0.92 | 0.80 | 0.79 |

phase. The resolution increases with decreasing methanol concentration. $R_F$ values are reported in Table I.

At a methanol concentration of 50% both the retention and the selectivity are satisfactory, so we used this composition in the analysis of a series of substituted

TABLE II

$R_F$ VALUES OF SOME SUBSTITUTED BENZOIC ACIDS

Solvent composition: methanol–0.1 *M* acetic acid (50:50).

| *Compound* | $R_F$ | $R_M^*$ | *Hansch constant* ($\pi$) |
|---|---|---|---|
| Benzoic acid | 0.27 | 0.43 | 0 |
| Vanillic acid (3-methoxy-4-hydroxybenzoic acid) | 0.55 | −0.08 | −0.16 |
| 4-Hydroxybenzoic acid | 0.66 | −0.29 | −0.30 |
| Anisic acid (4-methoxybenzoic acid) | 0.20 | 0.60 | 0.08 |
| 4-Chlorobenzoic acid | 0.11 | 0.91 | 0.87 |
| 3,5-Dinitrobenzoic acid | 0.48 | 0.034 | −0.10 |
| 4-Bromobenzoic acid | 0.07 | 1.10 | 0.98 |
| 4-Nitrobenzoic acid | 0.25 | 0.48 | 0.02 |
| 3-Hydroxybenzoic acid | 0.50 | 0.0 | −0.38 |

* $\xi = 1$.

TABLE III

RETENTION OF SOME SUBSTITUTED BENZOIC ACIDS IN DIFFERENT SOLVENTS

| *Compound* | *Hansch constant* ($\pi$) | $R_M^*$ | | | |
|---|---|---|---|---|---|
| | | *80:20*** | *60:40*** | *40:60*** | *50:50*** |
| Benzoic acid | 0 | −0.17 | 0 | 0.60 | 0.43 |
| 4-Chlorobenzoic acid | 0.87 | 0.28 | 0.47 | 1.12 | 0.91 |
| 3-Methoxy-4-hydroxybenzoic acid (vanillic acid) | −0.16 | −0.66 | −0.36 | 0.10 | −0.08 |
| 4-Methoxybenzoic acid (anisic acid) | 0.08 | −0.10 | 0.12 | 0.65 | 0.60 |
| 3,5-Dinitrobenzoic acid | −0.16 | −0.41 | −0.10 | 0.60 | 0.034 |
| 4-Nitrobenzoic acid | 0.02 | −0.23 | 0.17 | 0.82 | 0.48 |
| 4-Hydroxybenzoic acid | −0.30 | −0.69 | −0.50 | 0.034 | −0.29 |

* $\xi = 1$.

** Proportions of methanol and 0.1 *M* acetic acid in solvent.

benzoic acids. *Ortho*-substituted compounds were not selected in order to avoid any interference with a possible *ortho* effect. The results are summarized in Table II. It can be seen that hydroxy-containing acids are much less retained than benzoic acid, while nitro- or methoxy-substituted benzoic acids are more retained. 4-Chloro- and 4-bromobenzoic acids are even more strongly retained.

The retention data have been compared with the hydrophobic parameter $\pi$ as defined by Leo *et al.*[32]. The higher the value of $\pi$ the more retained is a substance, as shown in Tables II and III. The correlation coefficient varies from 0.67 to 0.83 (Table IV), which is only fair, but typical of this approach. This is to be expected as the Hansch constant is not sufficient to describe the retention behaviour of a molecule. Steric effects and the influence of the carbon skeleton on the molecular polarizability should also be taken into account[33].

TABLE IV

CORRELATION BETWEEN HANSCH CONSTANT AND RETENTION DATA

Correlation: $R'_M = a\pi + b$. For retention data, see Table III.

| *Parameter* | *Solvent composition** | | | |
|---|---|---|---|---|
| | *80:20* | *60:40* | *50:50*** | *40:60* |
| *a* | 0.80 | 0.74 | 0.95 (0.89) | 0.82 |
| *b* | −0.32 | −0.07 | 0.25 (0.25) | 0.52 |
| Correlation coefficient ($r^2$) | 0.82 | 0.75 | 0.74 (0.83) | 0.67 |

* Proportions of methanol and 0.1 *M* acetic acid.

** Values in parentheses are data from Table II.

## CONCLUSION

Excellent results have been obtained with the new plates available for reversed-phase chromatography. Their properties are in agreement with what could be expected from results obtained in HPLC.

The main problems are associated with the wettability of the plate by the solvent, preventing the use of water-rich mobile phases, and with detection making necessary the development of new spray reagents.

## ACKNOWLEDGEMENTS

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