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STRATEGIES OF MOBILE PHASE TRANSFER FROM THIN-LAYER TO MEDIUM-PRESSURE LIQUID CHROMATOGRAPHY WITH SILICA AS THE STATIONARY PHASE

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SUMMARY

The location of the fronts in thin-layer chromatography (TLC) with multi-component mobile phases is discussed with reference to the possibilities of mobile phase transfer via analytical overpressured-layer chromatography (OPLC) to preparative medium-pressure liquid chromatography (MPLC). The advantage of this procedure is that the mobile phase of the TLC separation where the substance zones are distributed over the whole R_F range can be transferred to preparative column chromatography without limitations. Because OPLC may be used as equilibrated or non-equilibrated planar column systems and all mobile-phase fronts may be seen, it can be applied as a pilot method for preparative MPLC. Depending on the results of the analytical OPLC separation, four possibilities exist for mobile phase transfer to MPLC; these are discussed.

The generally useful method is to equilibrate the dry-filled column (silica of TLC quality with an average particle size of 15 μm) with a solvent in which the substances to be separated do not migrate and which was used for the prerun in analytical OPLC. The separation is then started with the optimized TLC mobile phase where the substances are distributed over the whole R_F range. Transfer of the optimized TLC mobile phase via OPLC to MPLC is demonstrated by the separation of furocoumarin isomers from the roots of *Heracleum sphondylium* and the ginsenosides from *Panax ginseng* C. A. Meyer. The separation of anthraquinone aglycones from *Rhamnus frangula* is an example of the direct transfer of the mobile phase from TLC to MPLC.

INTRODUCTION

High-performance liquid chromatography (HPLC) is the best established method[1,2] for scale-up to different preparative column liquid chromatographic (CLC) methods. Thin-layer chromatography (TLC) is accepted in many laboratories as a rapid pilot method for preparative normal-phase (NP) CLC[3–7]. However, the disadvantage is that, for a successful preparative separation, the TLC separation

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should be obtained below $R_F = 0.3$[8]. It is almost impossible to achieve a separation in this small range for complex separation problems, and the result cannot always be predicted by using multi-component mobile phases.

In this paper we report different strategies for transfer of the optimized TLC mobile phase via analytical overpressured-layer chromatography (OPLC) to preparative NP medium-pressure liquid chromatography (MPLC)[9], using the whole R_F range (0.1–0.9) of the TLC plate for the analytical separation.

EXPERIMENTAL

The analytical separations were carried out on silica 60 F_{254} TLC Alufoils from Merck (Darmstadt, F.R.G.) in unsaturated chromatographic chambers. MPLC glass columns obtained from Labomatic (Schönenbuch, Switzerland) (735 × 26 mm I.D. and 584 × 37 mm I.D.) and from Büchi (Flawil, Switzerland) (460 x 36 mm I.D.) were dry-filled with TLC silica gel GF_{254} having an average particle size of 15 μm (Merck).

Analytical reagent grade solvents were used for all separations. The mobile phase was delivered with either a Labomatic MD-80/100 MPLC pump, a Lewa (Leonberg, F.R.G.) Lab M5 pump or a Büchi 681 chromatography pump.

The prepurified extract of furocoumarins was obtained from *Heracleum sphondylium*[10], the ginsenosides from *Panax ginseng* C. A. Meyer[11] and anthraquinone aglycones from a hydrolysed extract from *Rhamnus frangula*[12]. Samples were applied with a Linomat IV TLC spotter from Camag (Muttenz, Switzerland) for the analytical separations. For MPLC separations, sample injection was performed with a Rheodyne (Cotati, CA, U.S.A.) four-way valve.

For OPLC separations a Chrompres 10 apparatus (Labor-MIM, Budapest, Hungary) was used at 10 bar overpressure. For the one-directional linear OPLC separations all four sides of the plates were impregnated with Impres II polymer suspension (Labor-MIM). Two channels were scratched out at a distance of 18 cm for the solvent inlet and outlet.

The densitograms in the absorption mode were recorded at 313 nm for furocoumarins (mercury source lamp) and at 254 nm for the anthraquinone aglycones with a Camag TLC scanner II, coupled with an HP 9000-216 computer (Camag) and an SC-920 high-speed TLC scanner (Shimadzu, Kyoto, Japan). For visual detection of ginsenosides, 1% vanillin in sulphuric acid was used and the densitograms were recorded at 540 nm.

For the on-line preparative MPLC separations, an LKB (Bromma, Sweden) detector with a 313-nm filter for furocoumarins and a 254-nm filter for anthraquinone aglycones, coupled with an LKB 2210 recorder and an LKB 7000 Ultrarac fraction collector, was used.

RESULTS AND DISCUSSION

Theoretical aspects

The main difference between TLC and CLC is that TLC is a non-equilibrated system, whereas CLC is generally a system equilibrated with the mobile phase. The rule that the substances have to be separated below $R_F = 0.3$ in TLC for a successful

transfer to preparative CLC[8] is based on the fact that below R_F = 0.3 the TLC system is generally also equilibrated, meaning that all compounds migrate within the last front of a multi-component mobile phase (see Fig. 1a). In all theoretical cases shown in Fig. 1 the mobile phase consists of four solvents. The fronts (α, β, γ, δ) are rarely visible in TLC, except for the α-front. On transferring the mobile phase of the TLC separation shown in Fig. 1b to CLC, the compounds migrating between the γ and δ front will be eluted together, because the TLC solvent composition between these two fronts is not identical with the eluent of the equilibrated column. All other compounds could be separated under these conditions by CLC.

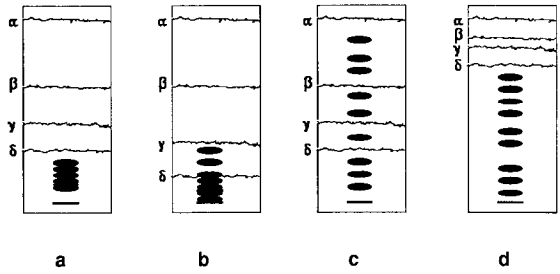

Fig. 1. Location of the fronts of a multi-component mobile-phase system and the compound zones in TLC (for explanation, see text).

On using the whole R_F range for the separation of the compounds in TLC, an ideal distribution, as depicted in Fig. 1c, is obtained, with all compounds separated. However, owing to the several fronts occurring between these compounds, a preparative CLC separation would not be possible with this mobile phase. A special case is shown in Fig. 1d, where all compounds migrate within the last front and are well separated. This mobile phase could be used for CLC separation and a good result would be obtained.

Because OPLC can be used as an equilibrated or non-equilibrated "planar column", this method may be employed as a pilot technique for the transfer of the optimized TLC mobile phase to the various CLC methods, *e.g.*, MPLC with silica as stationary phase. The plates must be prepared as described under Experimental. The optimized TLC mobile phase obtained from an unsaturated chromatographic tank cannot generally be transferred to analytical OPLC because gas and/or air are adsorbed on the surface of the stationary phase. This causes a disturbing zone[13] in the completely closed OPLC chamber, which distorts the substance zones within its range. The location of the disturbing zone depends on the mobile phase composition and pressure relationships, which are influenced primarily by the flow-rate.

As shown in an earlier study on the disturbing zone, three possibilities exist for transfer of the TLC mobile phase to analytical OPLC[14,15]:

(1) Variation of the flow-rate to either extremely high or low values to place the disturbing zone in an R_F range where it does not distort the separation.

(2) Reduction of the solvent strength of the mobile phase and further development. This method can be used only for the separation of closely related structures, where the reduction of the solvent strength does not result in a change in order of elution of consecutive compounds.

(3) A prerun with a suitable solvent, in which the applied samples do not migrate and which is miscible with the mobile phase. For non-polar compounds this solvent is usually *n*-hexane; for polar compounds such a solvent must be considered during the TLC mobile phase optimization[16]. The plate is developed with this solvent until no more air bubbles are eluted from the plate. The separation may then be started with the mobile phase.

Among these possibilities, the third is generally applicable to the transfer of the optimized TLC mobile phase to analytical OPLC. Because OPLC is used in this way as a specially equilibrated planar column, the OPLC mobile phase (including prerun) can also be transferred to preparative MPLC.

Mobile phase transfer to preparative MPLC

The mobile phase transfer strategy for preparative MPLC separation can be deduced from the results of the analytical OPLC experiments used as a pilot method. As the TLC/OPLC experiments are carried out on plates with an average particle size of 11 μm, silica with an average particle size of 15 μm is used for preparative MPLC. The column is dry-filled with the aid of a vacuum and nitrogen overpressure[9]. Theoretically, all transfer possibilities described above for OPLC can also be applied to preparative MPLC.

(1) A good preparative separation is problematic when starting with a dry column with an adequate mobile phase velocity, because the location of the disturbing zone is difficult to predict. This method can only be used when the substances to be separated are in the lower R_F ($R_F < 0.3$) range in the OPLC separation.

(2) The same effect can be achieved by reduction of the solvent strength (S_T) of the TLC mobile phase. The influence of the solvent strength reduction on the separation must always be checked in analytical OPLC. This method is applicable to the separation of compounds with different structures only in certain instances.

(3) The dry-filled column must be equilibrated with the solvent used in the OPLC prerun, and then the separation may be started with the TLC/OPLC mobile phase. This method can be used independently of the structures of the substances to be separated and the location of the fronts (see Fig. 1c). With this method a certain dilution of the mobile phase has to be considered for the first-eluted compounds ($R_F < 0.8$ in TLC). With on-line detection, a shift of the baseline may be observed owing to the different mobile-phase compositions between the various fronts. Substances migrating on or near these fronts give very sharp peaks, but this can also be predicted from analytical OPLC. However, these effects have almost no influence on the quality of the preparative separation.

(4) In the special case where all substances are spread over the whole R_F range, and migrate within the last front of the multi-component mobile phase (in both TLC experiments and analytical OPLC as well), the column can be equilibrated with the TLC mobile phase (see Fig. 1d). As a result, the preparative separation will be similar to that in analytical OPLC.

In all transfer possibilities mentioned above for the determination of the amount of sample for MPLC, the TLC plate wil be overloaded in analytical fully off-line OPLC until the densitometric resolution is no longer satisfactory. Calculation is performed approximately according to the amount of extract per unit volume of the stationary phase (*e.g.*, 6.5 ml for the TLC plate and 468 ml for the glass columns investigated for the separation of furocoumarins).

Separation of fucocoumarin isomers from Heracleum sphondylium roots

Separation of the five main furocoumarin isomers from *Heracleum sphondylium* is shown as a typical example of the separation of apolar compounds. Optimization was started with TLC preassays, where diethyl ether, ethyl acetate and chloroform were selected. Optimization with the regular part of the PRISMA model[16,17] gave the best separation at a solvent strength of S_T = 1.6 [ethyl acetate–chloroform–diethyl ether–*n*-hexane (8.8:17.6:17.6:56]. The densitogram of the TLC separation with this mobile phase is shown in Fig. 2a.

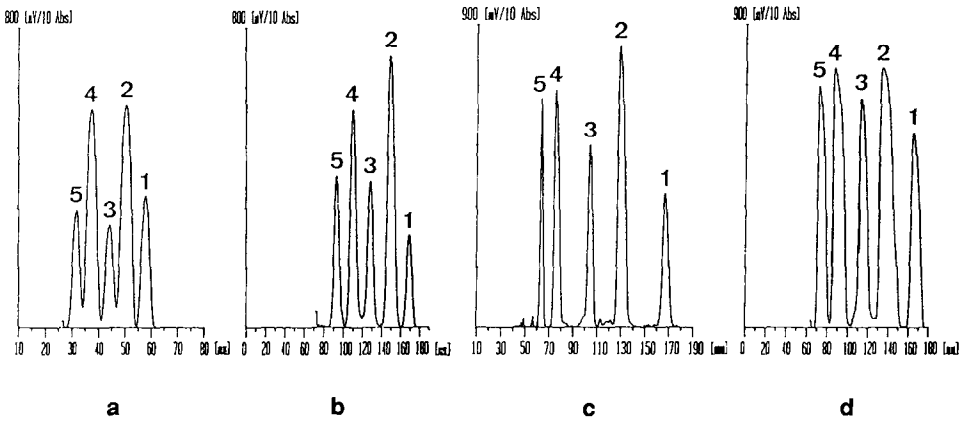

Fig. 2. Analytical TLC and OPLC separations of *Heracleum sphondylium* extract on TLC plates. 1 = Isobergapten; 2 = pimpinellin; 3 = bergapten; 4 = isopimpinellin; 5 = sphondin. (a) TLC separation in unsaturated chamber (separation distance, 8.5 cm); (b) OPLC separation after an *n*-hexane prerun; conditions, flow-rate 0.5 ml/min, development distance 18 cm, overrunning; (c) OPLC separation with reduced solvent strength (S_T = 1.0); (d) overloaded OPLC separation on TLC plate (volume. 6.5 ml) with a 4-mg sample and an *n*-hexane prerun.

The TLC mobile phase was transferred directly to analytical OPLC over an 18-cm separation distance. The adsorbed air or gas was eliminated either by a prerun with *n*-hexane (Fig. 2b) or by decreasing the solvent strength of the mobile phase to S_T = 1.0 (Fig. 2c). All five main compounds could be baseline separated by these methods. In the next step, under the same chromatographic conditions, the plate was overloaded to find the maximum amount of sample that could just be separated on a TLC plate by the OPLC technique (Fig. 2d).

In the next step, the mobile phase was transferred to MPLC. The results of the various mobile phase transfer procedures are shown in Figs. 3 and 4.

First, the separation was carried out with a dry-filled column to which the sample was applied. Separation was started with a mobile phase that was diluted with *n*-hexane, as an alternative method for eliminating adsorbed air and gas. Of course, the effectiveness of this procedure was tested first by analytical OPLC. The chromatogram of this MPLC separation is shown in Fig. 3.

Using the generally applicable method similar to analytical OPLC, the column was first equilibrated with *n*-hexane, then the sample was applied and the separation was started with the optimized TLC mobile phase. The resulting separation is depicted in Fig. 4a. Because in the analytical OPLC separation all compounds to be separated were migrating within the last front of the four-component eluent, the

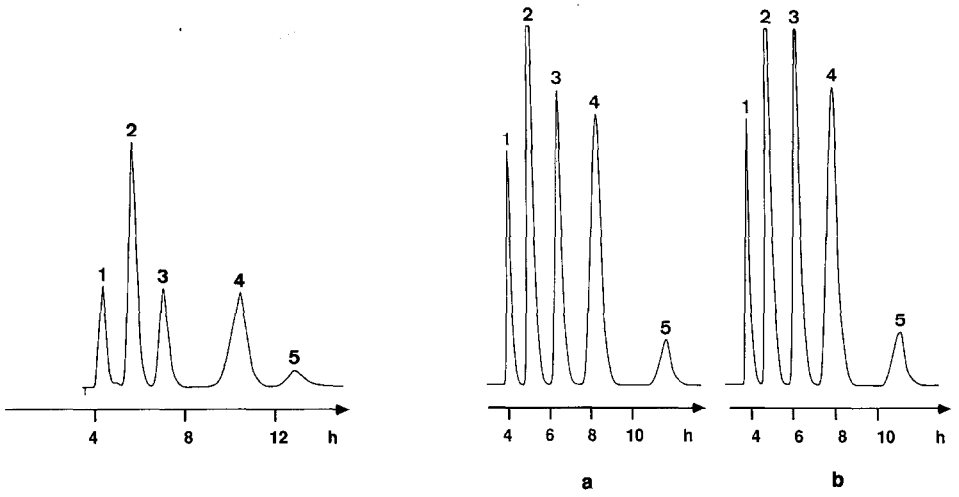

Fig. 3. MPLC separation of *Heracleum sphondylium* extract on non-equilibrated column. 1 = Isobergapten; 2 = pimpinellin; 3 = bergapten; 4 = isopimpinellin; 5 = sphondin. Column dimensions, 460 × 36 mm I.D. (468 ml); dry-filled with TLC silica gel GF_{254}; mobile phase, ethyl acetate–chloroform–diethyl ether–*n*-hexane (5.5:11:11:72.5); flow-rate, 5 ml/min; sample amount, 300 mg.

Fig. 4. MPLC separations of *Heracleum sphondylium* extract on an equilibrated column. 1 = Isobergapten; 2 = pimpinellin; 3 = bergapten; 4 = isopimpinellin; 5 = sphondin. Column dimensions: 460 × 36 mm I. D. (468 ml); dry-filled with TLC silica gel GF_{254}; TLC mobile phase, ethyl acetate–chloroform–diethyl ether–*n*-hexane (8.8:17.6:17.6:56); flow-rate, 5 ml/min; sample amount, 300 mg. (a) Equilibration with *n*-hexane; (b) equilibration with the TLC mobile phase.

separation of the furocoumarin-containing extract was also carried out on a column that had been equilibrated with the TLC mobile phase. The resulting chromatogram is shown in Fig. 4b.

On comparing the chromatograms obtained using the three mobile phase transfer possibilities, all methods are seen to give high-resolution separations. As expected, the longest separation time was observed with the lowest solvent strength, although the flow-rate was increased by 70%.

Equilibration of the column with *n*-hexane exerts a very small effect on the retention times, especially on those of the first-eluted compounds. Because all compounds migrated within the last front in OPLC, the chromatograms of the separation started with an *n*-hexane-equilibrated column or with a column equilibrated with the mobile phase are almost identical. As may be concluded from Figs. 3 and 4, the separation time could be reduced dramatically[18] by increasing the flow-rate, but this was not the aim of these experiments.

Separation of ginsenosides from Panax ginseng roots

Separation of the main ginsenosides (Rg2, Rg1, Rf, Re, Rd, Rc, Rb2, Rb1) from *Panax ginseng* is given as a typical example of the transfer of the optimized TLC mobile phase for polar compounds. From the TLC preassays in unsaturated chambers, methyl ethyl ketone, methanol and water were finally selected for further optimization of the mobile phase with the irregular top part of the PRISMA model[19,20].

In the next step, the optimized TLC mobile phase [methyl ethyl ketone–metha-

nol–water (70:22:8)] was transferred to analytical OPLC. The prerun was carried out with methyl ethyl ketone, in which the substance do not migrate. Then the separation was started with the mobile phase from the TLC separation. The densitogram of the analytical OPLC separation on a TLC plate is shown in Fig. 5a; the separation distance was 17 cm.

For the MPLC separation of the ginsenosides, the column, dry-filled with 15-μm silica of TLC quality, was equilibrated with methyl ethyl ketone. Thus, starting conditions were obtained that were similar to those in the OPLC separations. After the application of 150 mg of sample to a 73.5 × 2.6 cm I.D. column, the separation was started with the optimized mobile phase from the TLC separation. The eight main ginsenosides could be isolated almost without mixed fractions, as demonstrated in Fig. 5b.

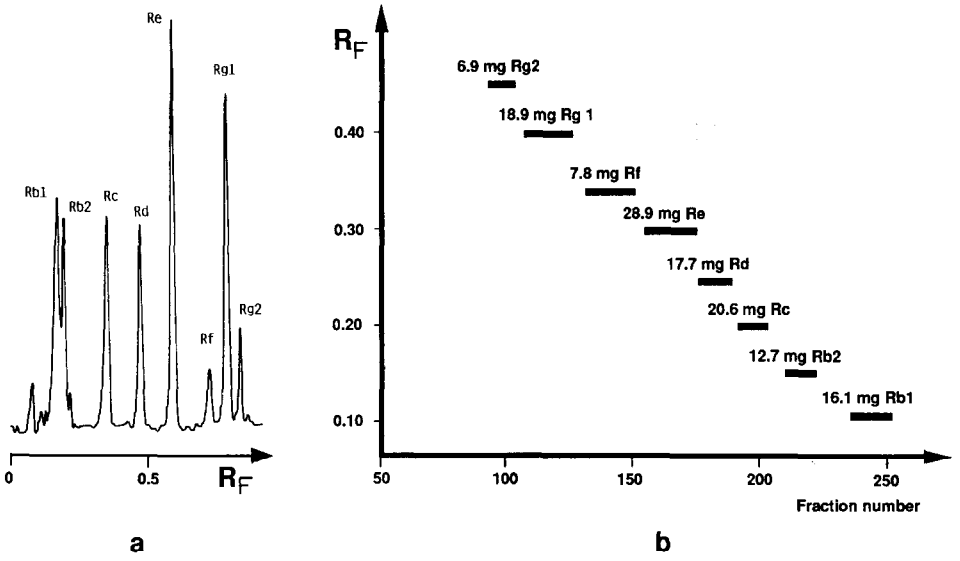

Fig. 5. Analytical OPLC and preparative MPLC separation of *Panax ginseng* extract. Mobile phase, methyl ethyl ketone–methanol–water (70:22:8) (a) OPLC separation on TLC plate after a methyl ethyl ketone prerun; conditions, flow-rate 0.5 ml/min; development distance 18 cm; (b) MPLC separation; column dimensions, 735 × 26 mm I.D. (410 ml), dry-filled with TLC silica gel HF_{254}; flow-rate 2 ml/min; sample amount 150 mg.

Separation of anthraquinone aglycones from Rhamnus frangulae cortex

The TLC separation of a prepurified, hydrolysed extract, containing three anthraquinone aglycones (chrysophanic acid, physcion and frangula emodin) was achieved in two steps: chrysophanic acid and physcion were separated with a mobile phase of tetrahydrofuran–*n*-hexane (1:7) (mobile phase A), as shown in Fig. 6a; then, to facilitate rapid migration of frangula emodin, tetrahydrofuran–*n*-hexane (3:7) (mobile phase B) was employed (see Fig. 6b).

This latter mobile phase was not suitable for the complete separation, as chrysophanic acid and physcion were not adequately resolved in this system. Therefore, the MPLC separation of the three compounds was carried out with a solvent strength

step gradient. The dry-filled (15-μm silica) column (584 × 37 mm I.D.) was first equilibrated with *n*-hexane. After the sample application, the separation was started with mobile phase A at a flow-rate of 9 ml/min. After elution of chrysophanic acid and physcion, the mobile phase was changed to B. The on-line MPLC trace is depicted in Fig. 6c. From a 1200-mg extract, 37.2 mg of chrysophanic acid, 48.4 mg of physcion and 819 mg of frangula emodin could be obtained.

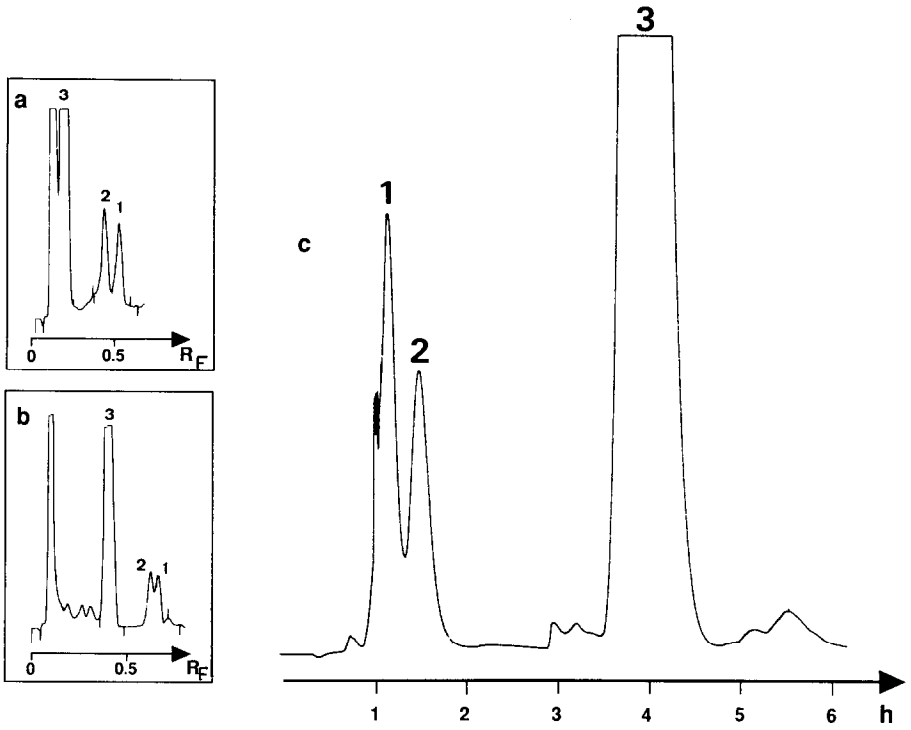

Fig. 6. Analytical TLC and preparative MPLC separation of anthraquinone aglycones. 1 = Chrysophanic acid; 2 = physcion; 3 = frangula emodin. (a) TLC separation in an unsaturated chromatographic tank with tetrahydrofuran–*n*-hexane (1:7); development distance, 9 cm. (b) TLC separation in an unsaturated chromatographic tank with tetrahydrofuran-*n*-hexane (3:7); development distance, 8.5 cm. (c) MPLC separation with a step gradient (for explanation see text); column dimensions, 584 × 37 mm I.D. (615 ml), dry-filled with TLC silica HF_{254}; flow-rate, 9 ml/min; sample amount, 1200 mg.

CONCLUSION

Four basic possibilities exist for mobile phase transfer from TLC to MPLC with silica as stationary phase. It is advantageous to use OPLC as a pilot method, because it is a forced-flow "planar-column" chromatographic system. OPLC separations may be carried out in both the fully off-line mode and the fully on-line mode[21,22], and both techniques can be applied to these transfer strategies. Transfer possibilities via analytical OPLC and directly from TLC to MPLC are summarized in Fig. 7.

If MPLC separation is carried out starting with a dry column, solvent strength reduction at a higher flow-rate is a good approach, provided that the influence of the

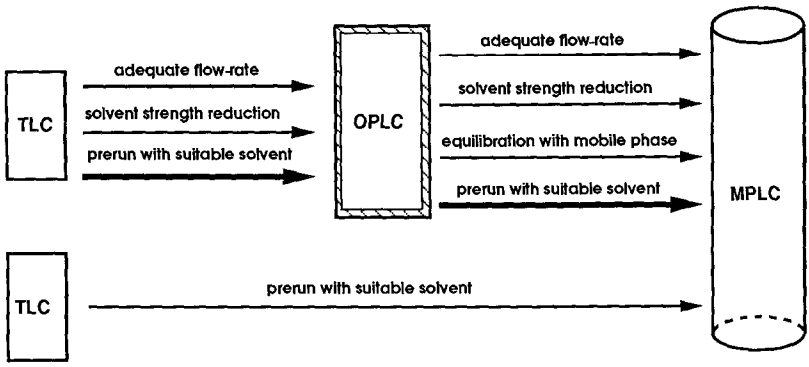

Fig. 7. Mobile phase transfer possibilities from TLC to MPLC with silica as stationary phase.

change of these parameters has been checked in analytical OPLC. This mobile phase transfer is possible if the correlation between solvent strength and R_F values is similar for all compounds to be separated. An extremely high mobile phase velocity can be employed only for the separation of non-polar compounds.

Equilibration of dry-filled MPLC columns with the optimized TLC/OPLC mobile phase is possible only if all compounds migrate within the last front of the multi-component eluent. This behaviour may be tested by analytical OPLC. Generally, the MPLC columns can be equilibrated with the solvent used for the prerun in analytical OPLC. This method is feasible regardless of the structures and properties of the substances to be separated. The solvent for the prerun is always *n*-hexane for non-polar compounds; for polar compounds, it must be decided during the mobile phase optimization process[8]. The compounds to be separated should not migrate in this solvent, and it should be either part of the mobile phase or at least miscible with the eluent.

The major advantage of the strategies presented is that mobile phase transfer starts from a TLC separation where the whole R_F range is used, in contrast to the general rule applied until now that all zones should be below $R_F = 0.3$ in TLC. However, prediction of the final MPLC result can be improved, especially when using the OPLC technique as a pilot method. Needless to say, these strategies can also be applied for the mobile phase transfer from TLC to all preparative column liquid chromatographic methods in which silica is used as the stationary phase.

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