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MOBILE PHASE OPTIMIZATION FOR MULTI-FUNCTIONAL SOLUTES IN LIQUID–SOLID CHROMATOGRAPHY

RESOLUTION OF SYNTHETIC INTERMEDIATES OF OLIGODEOXYRIBONUCLEOTIDES

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SUMMARY

The effects of mobile phase components on peak tailing and selectivity are examined for polar multi-functional solutes in liquid–solid chromatography on a silica gel adsorbent using various deoxynucleoside derivatives as sample solutes. Methanol is effective for suppressing peak tailing when combined with *n*-hexane as a diluent, but causes a decrease in the separation factor for pairs of deoxynucleoside derivatives. A combination of solvents is suggested for the diluent that will give an appropriate modification in separation factor so as to achieve maximum resolution of mixtures of polar multi-functional compounds.

INTRODUCTION

It is well known that liquid–solid chromatography (LSC) is suitable for separating compounds having different polar functional groups on a polar adsorbent and with a mobile phase composed of polar and non-polar solvents (normal phase system). According to statistical-mechanical analysis of LSC[1], a normal phase system is more effective for recognizing polar functions in organic molecules than a reversed-phase system comprised of a non-polar adsorbent and an aqueous mobile phase. Mobile phase components can be selected from a wide range of organic solvents, and the variation in the relative capacity ratio (separation factor) of a pair of solutes induced by changes in the mobile phase solvents (mobile phase selectivity effects) may be utilized for separating the solute pair. The optimization of selectivity[2] based on an interpretation of the selectivity effects in terms of the concept of "localization" suggested by Snyder *et al.*[3] may be useful in achieving maximum resolution. Furthermore, in preparative separations it is advantageous that most organic solvents can easily be removed from collected LSC fractions by evaporation.

In recent years, various multi-functional compounds related to biological constituents such as nucleotides and peptides have received increasing attention, and LSC has been used as a tool for the purification and preparation of such compounds.

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In the phosphotriester approach[4] to oligonucleotide synthesis, LSC is indispensable for the preparative separation of intermediate products. The application of a multi-solvent mobile phase[2] utilizing various kinds of selectivity effects may be more worthwhile for separating closely related multi-functional compounds, since the differences in particular functional groups between two similar solutes may result in smaller separation factors for multi-functional solutes than for monofunctional solutes. Our experience in oligodeoxynucleotide synthesis and other work, however, indicates that polar multi-functional compounds tend to have elution profiles with long tails, and thus a high separation factor may not always result in high resolution. It is necessary to reduce peak tailing and obtain sharp, symmetrical peaks in order to resolve a mixture of multi-functional solutes.

The tailing of observed chromatographic profiles is generally regarded as a modification of the Gaussian curve due to intra- and/or extra-column effects[5]. The intra-column peak tailing in LSC of polar solutes is usually attributed to non-linearity of the relevant adsorption isotherm or solute overloading of relatively high-energy sites on the adsorbent surface. Various highly polar solvents, including water, have been used as additives to the mobile phase to imporove the isotherm linearity[6]. Molecules of the polar solvent are assumed to be adsorbed preferentially by higher energy sites from which the non-linear isotherm originates and thus adsorption of the solute molecules onto such sites is prevented.

The asymmetric peak shapes for polar solutes, however, seem to persist in LSC even when the amount of solute injected is considerably reduced. This may suggest that tailing has a kinetic origin, *i.e.*, some kind of slow exchange of solute and solvent between the mobile and stationary phases is operative in the column. Solid surfaces are generally considered to have microscopic heterogeneity with respect to the activation energy for adsorption, and high activation energy sites may be assumed to exist on the surface of adsorbents used in LSC to give slow exchanges[5]. This type of tailing can be reduced by adding a polar solvent to the mobile phase if the molecules of the polar solvent are desorbed from the high activation sites slowly enough to obstruct adsorption of solute molecules. We selected intermediate products in oligodeoxynucleotide synthesis as sample solutes, and examined the effects of certain polar solvents on peak tailing.

The addition of a polar solvent to the mobile phase to reduce tailing may decrease retention and cause some change in selectivity for the sample mixture. Snyder *et al.*[3] have argued that the addition of a polar solvent which can be localized on the adsorption site of a polar adsorbent causes the largest decrease in retention for most strongly localized solutes . It has been also suggested that use of a more polar solvent as a mobile phase component may result in a smaller separation factor for a pair of polar solutes of similar adsorption area on the adsorbent surface[7]. Thus, even if a polar solvent component completely reduces peak tailing for multi-functional solutes, we cannot assert from the results alone that there will be any improvement in resolution. We examined the component solvents of the mobile phase for their selectivity effect as well as for their reducing effect on tailing and tried to find a way of increasing the separation factor for pairs of derivatives of deoxynucleosides while suppressing peak tailing. For maximum resolution of a complex sample mixture, mobile phases with modifiers were applied. Based on the results, we designed an effective multi-solvent mobile phase for resolving a mixture of polar multi-functional compounds in LSC.

EXPERIMENTAL

Reagents

The deoxynucleotide derivatives (intermediate products in the phosphotriester approach to oligodeoxynucleotides) were prepared from deoxynucleosides according to the literature[8]. The reagents for the preparation and the solvents were purchased from Wako (Osaka) and Aldrich (Milwaukee, WI, U.S.A.). The prepared derivatives were purified by silica gel LSC with a benzene–methanol mixture as the mobile phase. Sample purity was determined by thin-layer chromatography and nuclear magnetic resonance spectrometry.

Apparatus

A 250 × 4.6 mm I.D. column packed with 1.9 g of porous, spherical silica gel (average particle size 10 μm, average pore diameter 95 A, specific surface are *ca.* 380 m^2/g) (Kusano, Tokyo) was used. In a Model 5021 chromatograph (Varian, Palo Alto, CA, U.S.A.) two solvents were pumped alternately from the reservoirs, mixed in a 170 × 4 mm I.D. tube placed between the pump and column and introduced into the column. The column and mixing tube were thermostatted at 40°C and the ambient temperature was maintained at 24 ± 1°C. A 10-μl loop was used for injection. A UV detector SPD-1 with a 6.4-μl cell (path length 8 mm; Shimadzu, Kyoto) was used, the noise level being 10^{-7} a.u.

Procedure

Prior to analysis, the mobile phase was passed through the column for 1 h or more with the same flow-rate, 1 ml/min, as that for the analysis. The absorption at 280 nm was recorded, and the hold-up time, t_0, equivalent to the void volume of the column was determined by measuring the time from the injection of *n*-hexane to the first peak. The sample solutes were dissolved in chloroform and injected into the column. The capacity ratio, k', for each sample was determined from the elution time, t_R, for the peak maximum of the sample [$k' = (t_R - t_0)/t_0$].

RESULTS AND DISCUSSION

In the present study, the chromatographic peak shape was characterized by estimating the peak asymmetry factor[6], A_s, and relative peak sharpness[9], Q, as illustrated in Fig. 1. A high degree of peak tailing may be represented by high A_s values combined with low Q values.

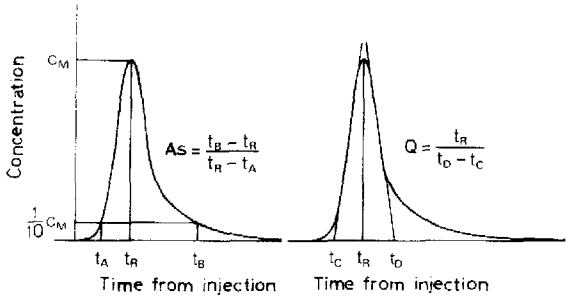

Fig. 1. Estimation of the peak asymmetry factor, A_s, and relative peak sharpness, Q.

The solutes chosen were 3'-5',O,N-tribenzoyldeoxycytidine and 5'-O-di-4-methoxytrityl-protected and unprotected N-benzoyldeoxycytidine and thymidine. The injected amount of each solute was adjusted to the range of the linear adsorption isotherm in which the A_s and Q values were independent of solute amount (10^{-6}–10^{-8} g per g of adsorbent). The effects of polar solvents on peak shape in LSC were first examined using a mobile phase consisting of the polar solvent and *n*-hexane, often chosen as a diluent in preparative LSC. The results are shown in Fig. 2. Because the mobile phase and therefore its polar component must have high elution strength to elute such polar samples, the following solvents were selected from polar solvents frequently used as polar components in preparative elution: ethyl acetate, tetrahydrofuran, dioxane and methyl, ethyl, isopropyl and *n*-propyl alcohols. The proportions of the mobile phase solvents was, in each case, experimentally determined when preparing the phase system in which all samples have capacity ratios within a reasonable range ($0.5 < k' < 10$).

When ethyl acetate, tetrahydrofuran or dioxane was used as the polar component the degree of tailing was so high and the peaks so spread out as to preclude

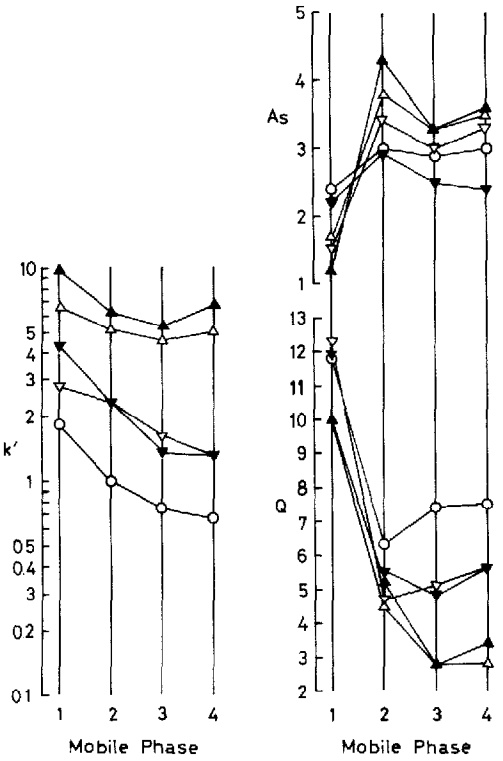

Fig. 2. Comparison of the ability of polar solvents to reduce peak tailing when used as the stronger components of a mobile phase in the LSC of deoxynucleoside derivatives on silica gel. Mobile phases: 1, *n*-hexane methanol (84:16, v/v); 2, *n*-hexane–ethanol (80:20); 3, *n*-hexane–2-propanol (70:30); 4, *n*-hexane–1-propanol (70:30). Samples: ▲, thymidine; ▼, 5'-O-di-4-methoxytritylthymidine; ○, N,3',5'-O-tribenzoyldeoxycytidine; △, N-benzoyldeoxycytidine; ▽, 5'-O-di-4-methoxytrityl-N-benzoyldeoxycytidine.

determination of A_s and Q with injected amounts in the range where the elution time at the peak maximum was independent of sample amount. These solvents are thus omitted from Fig. 2. The remaining polar solvents possess higher elution strengths than those omitted and this fact in itself suggests that solvents which interact strongly with the polar adsorbent and are slowly desorbed are effective for reducing sample peak tailing in LSC. It should be noted that in Fig. 2 the mobile phase containing methanol, the strongest solvent of the four alcohols, caused the greatest decrease in peak tailing (the lowest A_s and highest Q) and the most prolonged retention (see the plots of capacity ratio, k') for each sample. Thus, the ability of the mobile phase to reduce peak tailing is not proportional to its elution strength but dependent on its polar component. The values of A_s and Q obtained for the samples in the methanol-containing mobile phase were comparable to those for the alkyl esters of phthalic acid in *n*-hexane–ethyl acetate (9:1, v/v) as mobile phase, which exhibit relatively symmetrical peaks free from long tails, on the same column and under the same chromatographic conditions (for methyl, ethyl or *n*-butyl ester; $A_s = 1.6$, $Q = 11$). As mentioned previously, the long tails observed for a polar multi-functional solute in the range of sample size corresponding to the linear adsorption isotherm are considered to arise from the slow exchange of solute between the mobile and stationary phases. It may thus be concluded that methanol is capable of virtually preventing the sample solutes from participating in this slow exchange.

In Fig. 2, the capacity ratio, k', for each sample is plotted on a logarithmic scale for each mobile phase composition. The methanol-containing mobile phase yields differences in k' between the derivatives of thymidine (closed symbols) and between those of deoxycytidine (open symbols), all smaller than those obtained in mobile phases containing other alcohols, except for the difference between dimethoxytrityl-protected and unprotected deoxycytidine in the *n*-hexane–methanol system compared with that in the *n*-hexane–ethanol system. (In Fig. 2 the distance between two symbols plotted on a one-dimensional k'-coordinate system represents the logarithm of the separation factor and one can visually compare mobile phases in order to estimate the separation factor of a pair of solutes.) This may confirm that the use of a more polar solvent in the mobile phase in LSC decreases the separation factor of a pair of solutes of similar adsorption area[7]. Suppression of peak tailing by methanol as the stronger component of the mobile phase thus does not directly imply attainment of higher sample resolution.

However, it may also be expected that by decreasing the concentration of the stronger solvent in the mobile phase the separation factor value should increase for a pair of solutes of similar adsorption area[7]. We actually observed an increase in the separation factor with increasing retention for sample pairs on decreasing the methanol concentration in the *n*-hexane–methanol system. To adapt the mobile phase of decreased methanol content to a practical sample separation, a weakly polar diluent capable of supplementing the mobile phase elution strength is required in place of *n*-hexane. In this regard, we examined benzene, dichloromethane and chloroform. The mobile phases employed in Fig. 3 each contain one of these solvents and methanol in the appropriate proportion for a capacity ratio of about 10 for the unprotected-deoxyriboside sample most strongly retained in the column. By replacement of *n*-hexane with the other solvent, the methanol concentration required for elution of the samples decreases and the separation factors for each solute pair of the deriv-

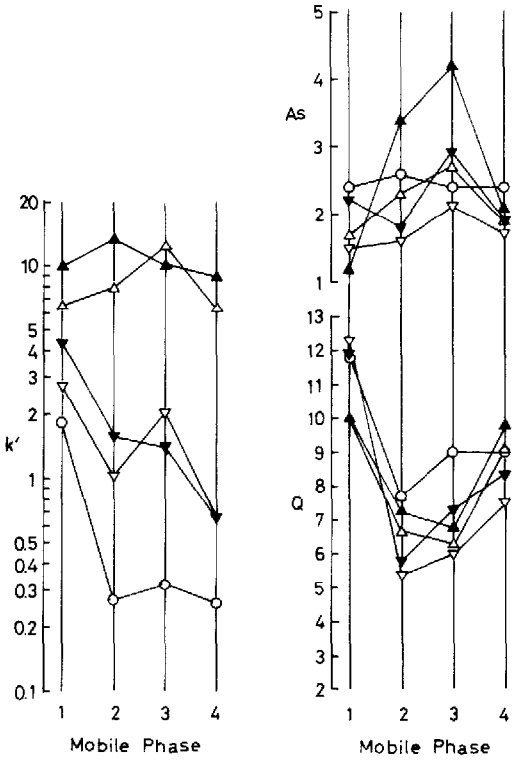

Fig. 3. Comparison of methanol-containing mobile phases with different diluents. Mobile phases: 1, *n*-hexane–methanol (84:16); 2, benzene–methanol (95:5); 3, dichloromethane–methanol (97:3); 4, chloroform–methanol (95:5). Samples as in Fig. 2.

atives of each deoxynucleoside considerably increase (about twice as much as those in *n*-hexane). The peaks obtained in the chloroform–methanol system are broader than those in the *n*-hexane-methanol system, but narrower and more symmetrical than those in a mobile phase comprising *n*-hexane and another alcohol from Fig. 2. Thus, in LSC for high resolution of multi-functional solutes, the following mobile phase may be suggested: as a stronger component, a highly polar solvent to suppress peak tailing and as a diluent, a solvent with appropriate elution strength so as to decrease the polar component concentration to such a degree that high separation factors are obtained for similar solutes.

For this method of selection of mobile phase components the available solvents (and therefore the selectivity) are limited. In order to utilize a wide variety of selectivity effects for the separation of a sample mixture, we tested various binary solvent mixtures as mobile phase diluents. Although by use of a binary mixture with benzene, dichloromethane or chloroform as diluent some selectivity intermediate between those of single-solvent diluents may be possible, we examined binary mixtures of *n*-hexane and a small amount of a polar solvent, by which a considerable range of selectivity may be expected. From a practical standpoint, a minimum amount of solvent was added to *n*-hexane to prepare a diluent that could be mixed with meth-

anol in any proportion at room temperature. The additives used were benzene, chloroform, ethyl acetate, tetrahydrofuran, dioxane and 2-propanol. The resulting ternary solvent mobile phases were examined for samples of deoxynucleoside derivatives in comparison with the mobile phases already tested. Fig. 4 shows that the ternary solvent mobile phases are similar to the chloroform–methanol mixture (Fig. 3) in giving relatively high Q and low A_s values for the samples, but give inferior separation factors. The differences in selectivity between the ternary solvent mobile phases appear in Fig. 4 as slight changes in the relative k' for each solute pair. Thus, modification of the mobile phase with additives does not lead to a wide variation in selectivity for the LSC of similar multi-functional compounds. However, we have found additive modification to be effective for obtaining favourable selectivity in the resolution of certain products of the phosphorylation of protected deoxynucleosides.

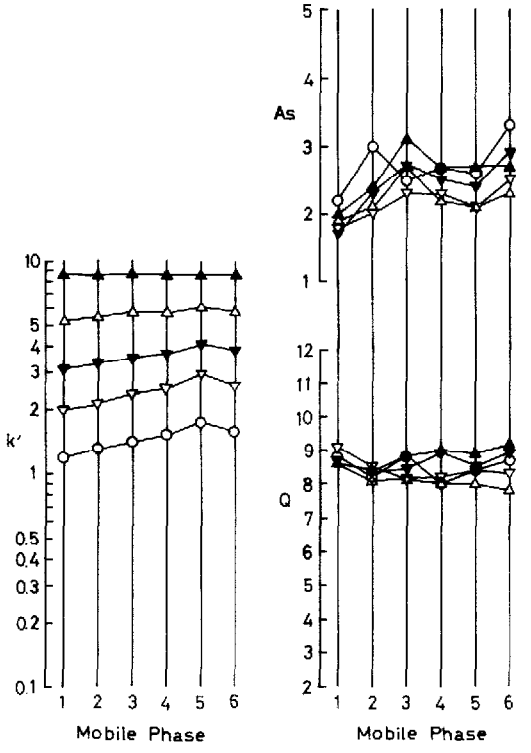

Fig. 4. Mobile phases with binary solvent diluents: 1, *n*-hexane–benzene (90:10)–methanol (85:15); 2, *n*-hexane–chloroform (96:4)–methanol (85:15); 3, *n*-hexane–ethyl acetate (95:5)–methanol (85:15); 4, *n*-hexane–tetrahydrofuran (96:4)–methanol (85:15); 5, *n*-hexane–dioxane (93:7)–methanol (87:13); 6, *n*-hexane–2-propanol (96:4)–methanol (87:13). Samples as in Fig. 2.

The chemical synthesis of oligodeoxynucleotides by the phosphotriester approach[4] involves phosphorylation of the porotected deoxynucleoside to obtain the protected monomers of deoxynucleotide triester, which can be polymerized to any oligodeoxynucleotide by repeated partial deprotection and coupling reaction. Although LSC has been used to purify these triester units, it may be difficult by LSC

completely to separate the remaining reactant (the protected deoxynucleoside) and by-products such as phosphotriesters that have two deoxynucleoside units from the required product due to their similar LSC retention and peak tailing properties. The sample mixture examined consisted of 5′-O-di-4-methoxytritylthymidine (a), diastereomers of 2-cyanoethyl 4-chlorophenyl 5′-O-di-4-methoxytritylthymidine-3′-phosphate (b_1, b_2) and 4-chlorophenyl 3′,3′-bis(5′-O-di-4-methoxytritylthymidine) phosphate (c). The mobile phases containing benzene, dichloromethane and chloroform respectively as the diluent did not yield the same selectivity (and elution order) for the sample mixture, but none of these mobile phases led to complete resolution when the upper limit of the capacity ratio of the sample mixture was set at 10.

Fig. 5 shows chromatograms obtained by applying ternary solvent mobile

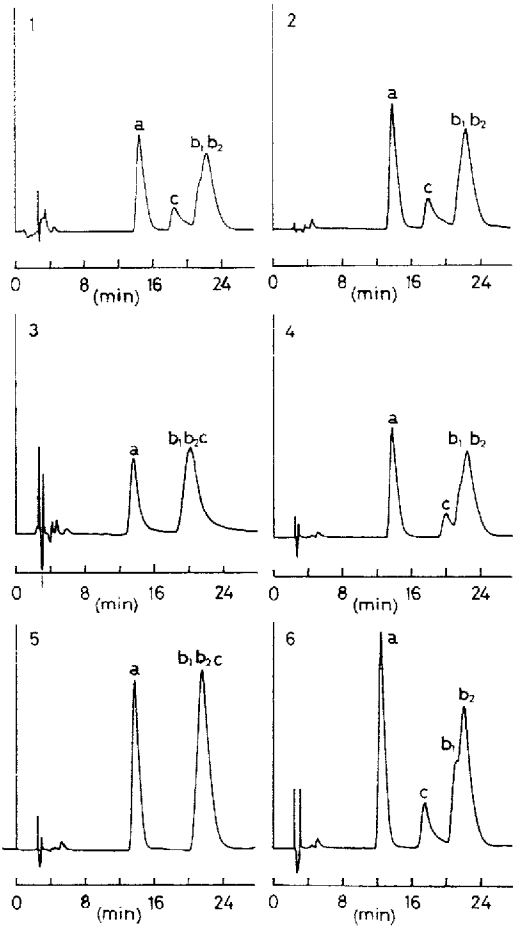

Fig. 5. Chromatograms of products of phosphorylation of protected thymidine, obtained by elution with ternary solvent mobile phases. Mobile phases: 1, (*n*-hexane–benzene)–methanol (92:8); 2, (*n*-hexane–chloroform)–methanol (90:10); 3, (*n*-hexane–ethyl acetate)–methanol (91:9); 4, (*n*-hexane–tetrahydrofuran)–methanol (90:10); 5, (*n*-hexane–dioxane)–methanol (91:9); 6, (*n*-hexane–2-propanol)–methanol (91:9). Composition of each diluent as in Fig. 4. Samples: a = 5′-O-di-4-methoxytritylthymidine; b_1, b_2 = diastereomers of 2-cyanoethyl 4-chlorophenyl 5′-O-di-4-methoxytritylthymidine-3′-phosphate; c = 4-chlorophenyl 3′,3′-bis(5′-O-di-4-methoxytritylthymidine) phosphate.

phases to the sample mixture of phosphotriesters. The ratio of methanol and the diluent in each mobile phase was experimentally determined for the most strongly retained solute in the sample mixture so as to give a capacity ratio of *ca.* 10. For this sample mixture, the differences in selectivity induced by changes in the additive were more apparent than those for the deoxynucleoside derivatives described above. The retention of solute c was more sensitively affected by changing the mobile phase composition than the other solutes. The statistical-mechanical theory of LSC[10] states that the degree of change in k' for a solute caused by a change in mobile phase composition is proportional to the effective adsorption area of the solute. In LSC with a polar adsorbent, the effective adsorption area for polymeric solutes consisting of unit polar parts may reflect the number of units in the solute molecule provided the individual units are arranged so that adsorption of one unit does not interfere with that of another unit. The sensitivity of solute c to a change in mobile phase composition may possibly be related to the fact that it is a dimeric nucleoside whereas the others are monomeric. By use of a series of such ternary solvent mobile phases the retention of solute c can be varied while keeping the retention of a, b_1 and b_2 almost constant. Almost complete resolution was attained using the *n*-hexane–chloroform–methanol or *n*-hexane–2-propanol–methanol system for purification of the phosphotriester products (b_1, b_2) with recovery of the reactant (a) and removal of the by-product (c).

In the synthesis of a polymeric compound from monomeric units, a problem frequently encountered is to resolve the complex mixture of products having different numbers of units. With polymers consisting of polar multi-functional units, such as nucleosides, the difference in the numbers of units in the products is reflected by differences in retention behaviour in normal phase LSC as discussed above. Differences in retention behaviour can be effectively applied to the resolution of complex mixtures of products formed in the synthesis of polar polymeric compounds provided that peak tailing is reduced and various mobile phase selectivity effects are usable in LSC. It may be realized through use of a mobile phase consisting of a polar solvent and which reduces tailing and a diluent that can be varied so as to achieve the required selectivity.

CONCLUSIONS

In LSC, intra-column peak tailing peculiar to polar multi-functional compounds can effectively be reduced through use of a strongly polar solvent, such as methanol, as the polar component of the mobile phase. When peak tailing is reduced, the availability of different selectivity effects of various solvents is the main problem in resolving a complex mixture of multi-functional solutes. The selectivity of the LSC system can be varied by use of different solvents to dilute the mobile phase. Through use of a weakly polar solvent such as chloroform or benzene as diluent, a relatively high separation factor is possible for pairs of similar solutes. A series of binary solvent diluents, such as a mixture of *n*-hexane and a small proportion of a polar solvent, is useful for attaining the required selectivity in resolution of complex mixtures.

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