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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A C_{18} POLYACRYLAMIDE-BASED PACKING

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SUMMARY

A rigid macroporous polyacrylamide-based packing having an octadecyl (C_{18}) chemically bonded phase has been developed for reversed-phase high-performance liquid chromatography. Separations of non-polar, polar, basic and acidic compounds are demonstrated with this packing and compared with the retention properties of a C_{18} silica packing and a polystyrene-based packing. The C_{18} polyacrylamide-based packing equilibrates rapidly to changes in the composition of the mobile phase and may be used at pressures up to $25 \cdot 10^6$ N/m^2. The packing retains its performance after prolonged contact with aqueous mobile phases at both low and high pH.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) is generally performed with a silica packing coated with an octadecyl (C_{18}) chemically bonded phase[1]. This column packing may be viewed as a hydrophilic polar support covered by a hydrophobic surface layer. Ideally, retention is dependent only on the bonded phase, but for some solutes, for example amines, interactions with residual silanol groups cause broadening or distortion of chromatographic peaks[2]. Improved surface coverage methods can reduce but may not eliminate peak-tailing for amines on C_{18} silica packings. A further limitation of bonded phase silica packings is the instability of the silica surface owing to possible dissolution when in contact with a mobile phase having a high pH[3].

In recent years there has been considerable interest in polymer-based packings for reversed-phase HPLC. Particular attention has been directed to polymers based on poly(styrene–divinylbenzene) (PS–DVB) because these packings are stable with eluents from pH 1–14[4–11]. Furthermore, amines often show little or no peak-tailing on PS–DVB packings compared with C_{18} silica packings[11,12]. Both the matrix and surface of PS–DVB packings are viewed as neutral non-polar PS. However, the presence of aromatic groups must generate somewhat different retention behaviour from

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that for C_{18} silica[10]. It is possible to bring the retention properties of PS-based packings closer to C_{18} silica by using a C_{18} bonded PS–DVB[13], but non-polar PS still constitutes the matrix. Somewhat more polar polymeric packings may be employed for specific separations by reversed-phase HPLC, see for example applications reported for polymethacrylate packings[14].

Hydrophobic interaction chromatography developed for biological macromolecules involves a soft gel filtration matrix in which hydrophobic groups (either alkyl or aryl) are covalently attached to a hydrophilic (wettable) gel such as agarose or cross-linked dextrans[15,16]. Unfortunately, gel filtration matrices have poor mechanical stability at high column pressures and so are unsuitable for HPLC. Improved rigidity is characteristic of TSK-Gel, type PW (based on a hydroxylated polyether)[17,18] which has been widely used for aqueous high-performance size-exclusion chromatography (HPSEC), and this type of gel may be modified for hydrophobic interaction chromatography[19].

Reversed-phase HPLC with C_{18} silica has become an extremely popular analytical technique for a wide range of compounds[20]. If a polymer-based packing having long-term stability is to replace C_{18} silica in separations requiring eluents at high pH, then these two types of packings must have similar retention properties. We believe that the most likely replacement (by analogy both with reversed-phase HPLC with silica and hydrophobic interaction chromatography) will result from a packing constituted from a hydrophilic polar polymeric support covered with a hydrophobic surface-layer. This approach is evident in the development of a C_{18} bonded phase on a vinyl acohol copolymer packing for reversed-phase HPLC[21,22]. In previous papers[23,24] we have reported the preparation of cross-linked polyacrylamide microspheres, which are both rigid and macroporous, and demonstrated their use for aqueous HPSEC. We have produced a packing having a C_{18} stationary phase bound to a polyacrylamide-based support, and brief chromatographic details for this packing were reported in a previous paper[25] which emphasised column efficiency data for the PS–DVB packing known as PLRP-S (tradename of Polymer Labs.). In the present paper reversed-phase HPLC separations are demonstrated with this C_{18} polyacrylamide-based packing. A major aim was to compare the retention characteristics for non-polar, polar, basic and acidic compounds for this packing with those of a C_{18} silica packing. Variations in the composition of the mobile phase are investigated and studies of the stability of the packing at low and high pH are reported.

EXPERIMENTAL

The polymeric column packing for reversed-phase HPLC is a polyacrylamide-based packing containing N-octadecylamide groups. Macroporous polyacrylamide particles were produced in an inverse suspension process by co-polymerising acrylamide and N,N′-methylenebisacrylamide with the cross-linking agent as the major monomeric component. The inverse suspension polymerisations were performed according to the conditions described elsewhere[26]. The bonded phase was prepared by first forming strongly nucleophilic amide anions on the surfaces of the porous polyacrylamide particles by the use of strong base[27], followed by treatment with *n*-octadecyl bromide. The polymer particles were separated by air classification, and optical and scanning electron micrographs indicated spherical particles with a narrow parti-

cle size distribution with a mean particle diameter of 10 μm. The mean pore diameter estimated from results obtained by HPSEC[25] was about 100 Å. The dry polyacrylamide-based particles were dispersed in methanol, and this slurry was packed into a column (150 × 4.6 mm I.D.) at pressures up to 3500 p.s.i. (1 p.s.i. ≡ 6894.8 N/m^2. The performance of this column was compared with a column (100 × 4.6 mm I.D.) containing 5 μm Hypersil ODS 100 Å supplied by Hewlett-Packard (Avondale Division, Avondale, PA, U.S.A.). HPSEC separations indicated that the two packings had similar pore size distributions[25].

The chromatographic apparatus consisted of a Knauer pump Model 64 (Dr. Herbert Knauer, Berlin, West Germany), a Rheodyne Model 7125 injection valve (20 μl loop), supplied by HPLC Technology (Macclesfield, U.K.) a thermostatted oven (Applied Chromatography Systems) supplied by HPLC Technology and a Pye Unicam variable-wavelength UV detector Model LC3 operated at 254 nm, supplied by Pye Unicam (Cambridge, U.K.). Injection volumes were 5 μl and solute concentrations were typically below 40 $\mu g/cm^3$. Analytical grade solutes and reagents (Fisons Scientific Equipment Division, Loughborough, U.K.) and doubly distilled water were used throughout this work. Separations were performed at ambient temperature with an eluent flow-rate of 0.5 cm^3/min. Separations of caffeine, theophylline, benzoic acid and toluic acid were performed at 37°C with acetonitrile–10 m*M* sodium acetate pH 4.5 (20:80, v/v) as eluent. The composition of the eluent in separations with mixtures of methanol and water is given in tables and figures. Data for capacity factor k' were derived from $(t_R - t_0)/t_0$, where t_R is the retention time for the solute and t_0 is the time for an unretained peak obtained by injecting a liquid mixture with a volume composition different from that of the eluent, for example methanol–water (10:90) for the eluent methanol–water (70:30). Values for separation factor (or selectivity, α) for two solutes 1 and 2 were determined from k'_2/k'_1.

RESULTS AND DISCUSSION

Retention behaviour

Chromatograms for non-polar aromatic compounds are shown in Fig. 1. Derived values for k' for toluene, naphthalene and anthracene are given in Table I.

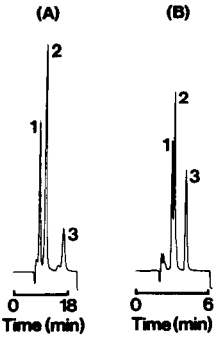

Fig. 1. Separation of non-polar aromatic compounds with (A) C_{18} polyacrylamide-based packing and (B) Hypersil ODS packing. Peaks: 1 = toluene, 2 = naphthalene, 3 = anthracene. Eluent: methanol–water (90:10, v/v) at ambient temperature.

TABLE I

CAPACITY FACTORS FOR ELUTIONS WITH METHANOL–WATER (90:10, v/v) AT AMBIENT TEMPERATURE

Solute	*k'*	
	Polyacrylamide-based packing	*Silica packing*
Toluene	0.28	0.45
Naphthalene	0.54	0.54
Anthracene	1.33	0.96
Biphenyl	0.70	0.67
Terphenyl	1.71	1.50

These results indicate similar behaviour for the polyacrylamide-based and silica column packings. These two column packings also produce similar separations of biphenyl and terphenyl, as shown by the chromatograms in Fig. 2 and the calculated values of k' in Table I. Data for α are shown for terphenyl and biphenyl in Table II, confirming excellent resolution of these two solutes with the polyacrylamide-based packing incorporating a C_{18} bonded phase.

Chromatograms for two polar alkyl phthalates are shown in Fig. 3. The derived values of k' for diethyl phthalate and dibutyl phthalate in Table III indicate that the two column packings produce somewhat different separation behaviour. This is further confirmed by the values of α in Table II. Although the selectivity for the C_{18} polyacrylamide-based packing is not as high as for the column of C_{18} silica, the column containing the C_{18} polyacrylamide-based packing provides a baseline separation of the two alkyl phthalates under the operating experimental conditions.

Chromatograms for basic compounds are shown in Fig. 4. The excellent baseline separation of pyridine and aniline with the C_{18} polyacrylamide-based packing is

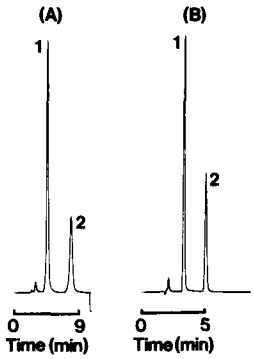

Fig. 2. Separation of non-polar aromatic compounds with (A) C_{18} polyacrylamide-based packing and (B) Hypersil ODS packing. Sample: 1 = biphenyl, 2 = terphenyl. Eluent: methanol–water (90:10 v/v) at ambient temperature.

TABLE II

SELECTIVITIES

Solutes	α	
	Polyacrylamide-based packing	*Silica packing*
Terphenyl–Biphenyl	2.43	2.24
Dibutyl phthalate–diethyl phthalate	5.13	6.26
Toluic acid–benzoic acid	3.01	2.66

confirmed by the values of k' given in Table III. The C_{18} polyacrylamide-based packing together with an eluent containing 70% methanol produces peaks in Fig. 4 which are symmetrical and which correspond to efficiencies similar to those for non-polar solutes. Chromatograms for the C_{18} silica packing in Fig. 4 show a reversal in elution order of the two solutes compared with the C_{18} polyacrylamide-based packing. The broadening and tailing of the peak for pyridine with the column of C_{18} silica packing may arise from solute interaction with residual surface silanol groups. For basic compounds interactions with residual surface silanol groups may influence retention[11,28]. For example, the capacity factor ($k' = 0.25$) for caffeine with the C_{18} polyacrylamide-based packing was somewhat below $k' = 1.0$ for the C_{18} silica packing, and the selectivity ($\alpha = 1.35$) determined with caffeine and theophylline was lower than 1.65 for the C_{18} silica packing. Whilst the separation mechanism with the C_{18} polyacrylamide-based packing here should be simpler than a mixed-mode separation occurring with a bonded phase silica, a mixed mechanism of retention might occur for other types of compounds with the C_{18} polyacrylamide-based packing.

Chromatograms for acidic compounds are shown in Fig. 5. Excellent resolution of benzoic acid and toluic acid with the C_{18} polyacrylamide-based packing is obtained,

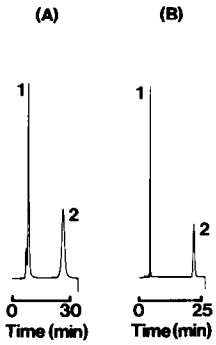

Fig. 3. Separation of polar alkyl phthalates with (A) C_{18} polyacrylamide-based packing and (B) Hypersil ODS packing. Peaks: 1 = diethyl phthalate, 2 = dibutyl phthalate. Eluent: methanol–water (70:30, v/v) at ambient temperature.

TABLE III

CAPACITY FACTORS FOR ELUTIONS WITH METHANOL–WATER (70:30, v/v) AT AMBIENT TEMPERATURE

Solute	*k'*	
	Polyacrylamide-based packing	*Silica packing*
Diethyl phthalate	0.67	1.00
Dibutyl phthalate	3.42	6.26
Pyridine	0.21	0.77
Aniline	0.58	0.28

as confirmed by the derived values of k' given in Table IV. The C_{18} polyacrylamide-based and C_{18} silica packings have similar values for α, as shown in Table II.

Mobile phase composition

In reversed-phase HPLC, the solute is distributed between a non-polar stationary phase (bonded C_{18}) and a polar mobile phase, so elution will be very dependent on the organic content of the mobile phase. Results for k' for non-polar aromatic compounds in eluents consisting of mixtures of methanol and water are displayed in Fig. 6. For both types of column packing it is evident that increasing the organic content of the mobile phase reduces k', so the analysis time may be lowered by raising the proportion of methanol in the eluent. The same trend was observed for separations of polar alkyl phthalates and basic compounds with the C_{18} polyacrylamide-based packing as shown in Figs. 7 and 8.

Studies of the mobile phase composition illustrate how the retention properties of the C_{18} polyacrylamide-based packing are quite different from PS–DVB stationary phases. The data for k' for methanol–water mixtures as eluents given in Tables V and VI are always considerably higher for the PS–DVB packing (PLRP-S) than for the

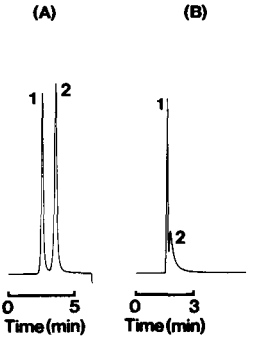

Fig. 4. Separation of basic compounds with (A) C_{18} polyacrylamide-based packing (peaks: 1 = pyridine, 2 = aniline) and (B) Hypersil ODS packing (peaks: 1 = aniline, 2 = pyridine). Eluent: methanol–water (70:30, v/v) at ambient temperature.

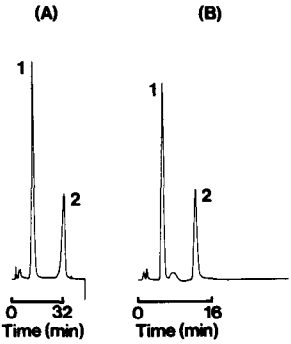

Fig. 5. Separation of acidic compounds with (A) C_{18} polyacrylamide-based packing and (B) Hypersil ODS packing. Peaks: 1 = benzoic acid, 2 = toluic acid. Eluent: acetonitrile–10 m*M* sodium acetate, pH 4.5 (20:80, v/v) at 37°C.

C_{18} polyacrylamide-based packing (Tables I and III). It has been noted previously that methanol has poor elution strength for PS–DVB column packings with methanol–water mixtures as eluents[29]. Data for k' for compounds in Table VI demonstrate that retention for the C_{18} polyacrylamide-based packing with methanol–water as eluent is similar to PLRP-S with acetonitrile–water (90:10, v/v), but in Table V other solutes separating with these two stationary phase–mobile phase combinations exhibit very different retention behaviour. Benzoic acid and toluic acid (Table IV) were not eluted from the PLRP-S column with these mobile phases. It must be concluded that these two polymer-based packings require quite different eluent compositions in order to obtain similar solute retention times.

Column packing stability

Macroporous polymer-based packings may undergo limited swelling in contact with some liquids. Swelling tests on C_{18} polyacrylamide-based packings indicated that particle volumes were very similar for methanol, methanol–water (from 10:90 to 90:10, v/v), and acetonitrile. Therefore, a column packed using methanol may be flushed immediately with the required mixture of methanol and water. It is not necessary to flush the column with mixtures having intermediate compositions. Rapid equilibration was proved by observing reproducible chromatograms on repeated in-

TABLE IV

CAPACITY FACTORS FOR ELUTIONS WITH ACETONITRILE–10 m*M* SODIUM ACETATE pH 4.5 (20:80, v/v) AT 37°C

Solute	k'	
	Polyacrylamide-based packing	*Silica packing*
Benzoic acid	2.24	1.60
Toluic acid	6.74	4.25

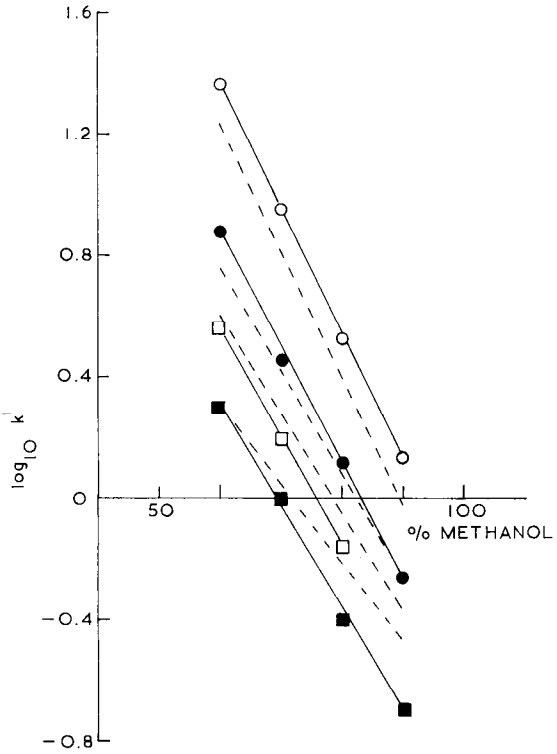

Fig. 6. Dependence of capacity factor on the composition of the eluent for C_{18} polyacrylamide-based packing (——) and Hypersil ODS packing (----). (○) Anthracene, (●) naphthalene, (□) toluene, (■) benzene.

jection of a test mixture of alkyl phthalates, when the data in Fig. 8 were again reproducibly obtained and so the C_{18} polyacrylamide-based packing equilibrates quickly to changes in mobile phase composition in separations operating by gradient elution. The degree of particle swelling will also determine the maximum pressure limit (and therefore fastest eluent flow-rate) for column operation. The packing pressure of $25 \cdot 10^6$ N/m^2 should not be exceeded because particle distortion and variations in close packing of particles may arise, resulting in a fall in column efficiency.

Column lifetime will be determined by the stability of the bonded phase in contact with a range of mobile phases. The column containing the C_{18} polyacrylamide-based particles was subjected to a series of experiments involving prolonged contact with methanol, water, and aqueous mobile phases at both low and high pH. After each experiment, column performance was assessed with a test mixture of three alkyl phthalates which were eluted isocratically with methanol–water (65:35, v/v).

After completing HPLC analyses, it may be necessary to clean up a reversed-phase packing by removing non-polar solutes adsorbed on the stationary phase. This may be achieved by flushing out the column with only the organic component of the mobile phase. The column containing the C_{18} polyacrylamide-based packing is extremely stable under these conditions. Thus, it was observed that the peak shapes,

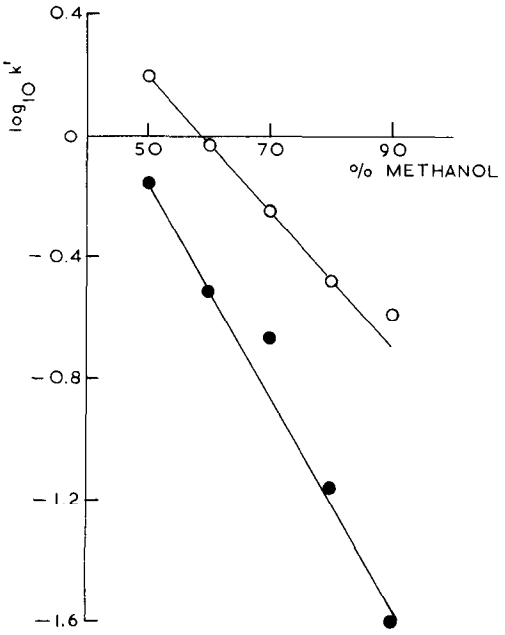

Fig. 7. Dependence of capacity factor on the composition of the eluent for C_{18} polyacrylamide-based packing. (○) Aniline, (●) pyridine.

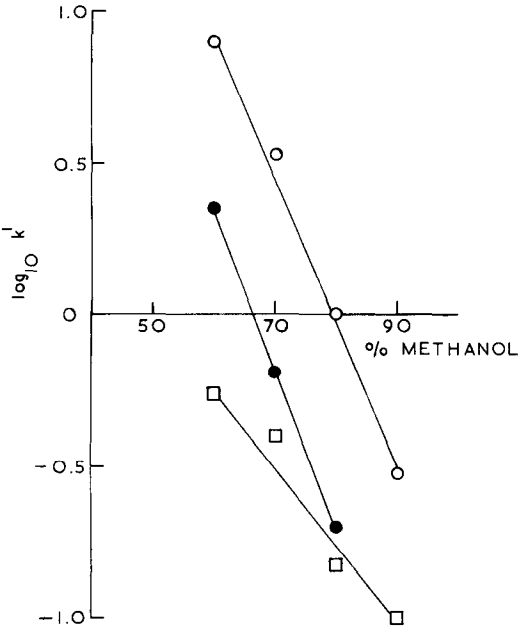

Fig. 8. Dependence of capacity factor on the composition of the eluent for C_{18} polyacrylamide-based packing. (○) Dibutyl phthalate, (●) diethyl phthalate, (□) dimethyl phthalate.

TABLE V

CAPACITY FACTORS FOR ELUTIONS WITH POLYMER-BASED PACKINGS AT AMBIENT TEMPERATURE

Solute	*k'*		
	*Polyacrylamide-based packing**	*PLRP-S**	*PLRP-S***
Toluene	0.28	4.95	1.21
Naphthalene	0.54	> 10	2.86
Anthracene	1.33	> 10	8.10
Biphenyl	0.70	> 10	1.62
Terphenyl	1.71	> 10	10.52

* Methanol–water (90:10, v/v) as eluent.
** Acetonitrile–water (90:10, v/v) as eluent.

TABLE VI

CAPACITY FACTORS FOR ELUTIONS WITH POLYMER-BASED PACKINGS AT AMBIENT TEMPERATURE

Solute	*k'*		
	*Polyacrylamide-based packing**	*PLRP-S**	*PLRP-S***
Diethyl phthalate	0.67	> 10	0.72
Dibutyl phthalate	3.42	> 10	1.76
Pyridine	0.21	1.21	0.28
Aniline	0.58	2.22	0.41

* Methanol–water (70:30, v/v) as eluent.
** Acetonitrile–water (90:10, v/v) as eluent.

TABLE VII

COLUMN CONDITIONS AT LOW AND HIGH pH FOR C_{18} POLYACRYLAMIDE-BASED PACKING

Aqueous eluent (0.1 mol/dm³)	*Temperature (°C)*	*Time (h)*	*Flow-rate (cm³/min)*
Hydrochloric acid	Ambient	7	0.5
Sodium hydroxide	Ambient	16	0.1
Sodium hydroxide	Ambient	24	0
Hydrochloric acid	50	16	0.1
Sodium hydroxide	40	24	0.1

capacity factors and efficiencies for all three solutes in the test mixture were the same before and after flushing the column with methanol. The same tests were also performed after flushing the column with distilled water, with the same results, thus confirming the excellent stability of the bonded phase in aqueous media.

Reversed-phase packings based on silica are generally employed with aqueous eluents having a pH in the range 3.5–7.5. The stability of the C_{18} polyacrylamide-based packing was examined with aqueous eluents having low and high pH. The experimental conditions are shown in Table VII. After each experiment, the column was flushed with methanol–water (65:35, v/v), and the test mixture was injected to establish column performance. Again, there was no change in peak shape and efficiency before and after exposure to either a low pH or high pH eluent, indicating no deterioration in the column packing. Therefore, the C_{18} polyacrylamide-based packing significantly extends the operating pH range in reversed-phase HPLC.

CONCLUSIONS

These initial results indicate that the C_{18} polyacrylamide-based packing is promising for reversed-phase HPLC. The data for capacity factor and selectivity for non-polar and polar solutes confirm that this packing operates by the reversed-phase mechanism, with similar results for retention and selectivity for aromatic and polar organic solutes to a conventional silica-based packing such as Hypersil ODS. The C_{18} polyacrylamide-based packing could therefore be considered as a direct replacement for a C_{18} silica packing with the additional advantage of providing long term physical and chemical stability over a wide pH operating range (1–13). The C_{18} polyacrylamide-based packing is particularly advantageous for nitrogen-containing bases, such as pyridine and aniline. Reversed-phase silica packings may generate broad tailing peaks for such basic compounds. The retention behaviour of the C_{18} polyacrylamide-based packing which like Hypersil ODS may be viewed as a hydrophobic layer on a hydrophilic rigid support is quite different from polystyrene-based packings such as PLRP-S.

We have reported the promising behaviour of the C_{18} polyacrylamide-based packing with simple test mixtures in typical reversed-phase separations and further work is now required on studies of more complex mixtures. This will have to be performed in conjunction with synthetic work on further column packings in order to optimise capacity factors and selectivities in relation to pore size distribution and mean pore diameter and to maximise column resolution in relation to packing particle diameter (*ca.* 5 μm).

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