

THE HPLC ANALYSIS OF POLAR ANALYTES WITH AQUEOUS MOBILE PHASES

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BACKGROUND and OBJECTIVES

The use of reversed phase HPLC materials with up to 100% aqueous mobile phases can often cause problems of long equilibration times, reduced and irreproducible retention times, poor peak shape and reduced quantitative reproducibility. This can occur at organic levels of less than 3-5%. The problem has been addressed by the development of special phases that can be operated for long periods in 100% water or aqueous buffers. This has traditionally been explained by a ligand folding or matting effect. The long alkyl chains – often C18 – are very hydrophobic and fold over each other trapping buffer and organic modifier. This accounts for the often long equilibration times of traditional materials when used in this fashion. In addition, the amount of accessible alkyl chain available to interact with analyte molecules is reduced – accounting for the reduction in retention and also in the rate of mass transfer. This latter factor leads to broadening of the peak, a poorer peak shape and reduced resolution.

A range of conventionally bonded, standard and new generation HPLC column materials, including C18, C8, C4, C1 and CN from several manufacturers were evaluated together with “high aqueous” RP products. The effect of end-capping was also determined. The parameters of retention factor, peak efficiency and peak shape were studied together with equilibration times using polar test probes such as carboxylic acids. Neutral and basic species were also investigated to determine the influence of the nature of the analyte on performance variation. This data was compared to the surface coverage and percentage carbon figures for the specific stationary phase to determine, qualitatively at least, the degree of “matting” of the stationary phase. The effects of column pressurisation were also briefly investigated to determine the degree of ligand folding or hydrophobic exclusion.

INTRODUCTION

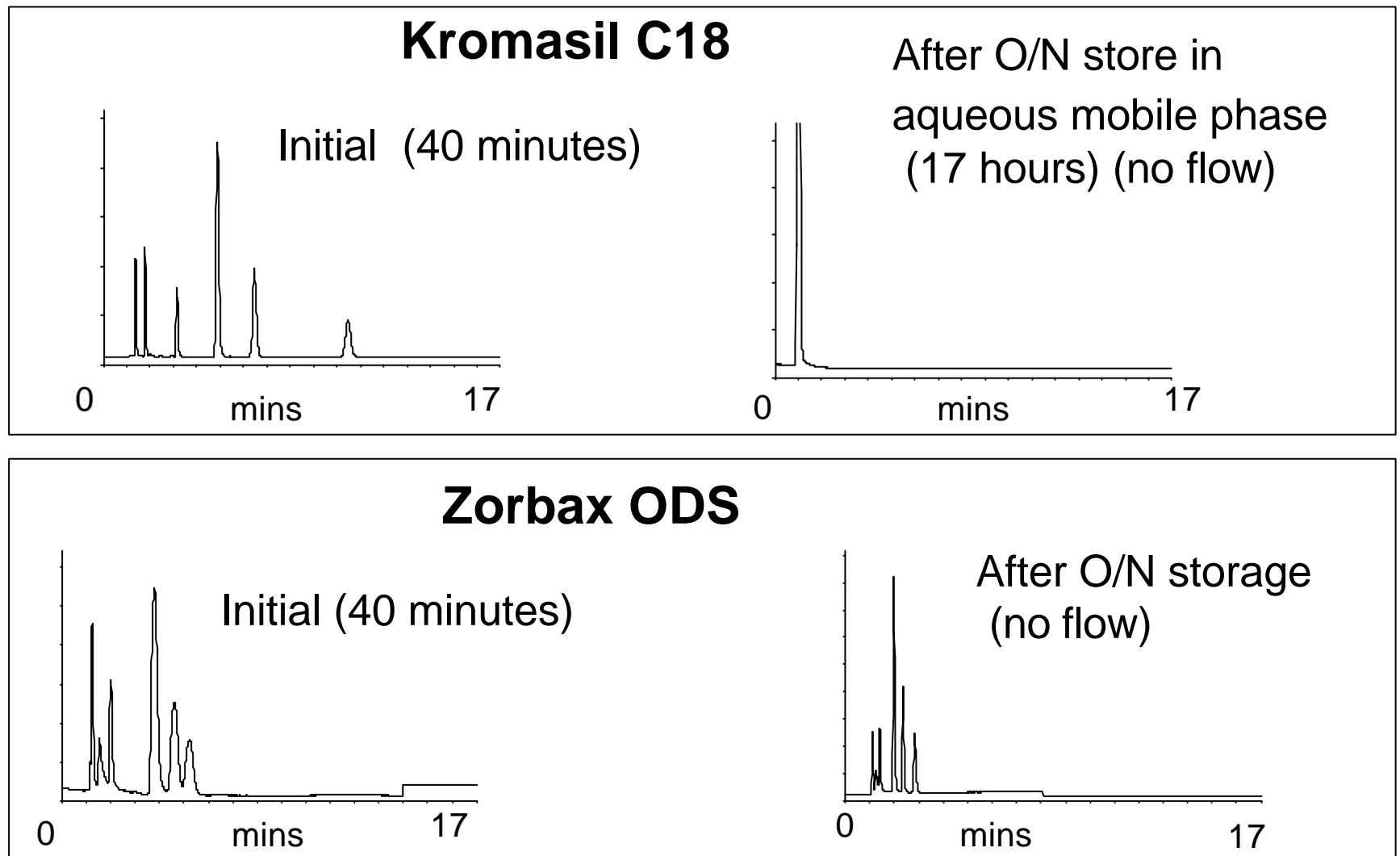
There are basically 3 types of scenario with respect to the applicability of reversed phase materials to use with high aqueous mobile phases. To use the nomenclature of other workers (e.g. R.A. Henry et al of Keystone Scientific and M. Przybyciel et al of ES Industries) these are:-

- Inert** - material can be used in 100% aqueous mobile phases with no deleterious effects.
- Resistant** - material can be used for short periods, up to a day, say, before regeneration, where a longer equilibration period or where some retention time drift can be tolerated.
- Prone** - phase collapses and material is not suitable for this application.

Fig. 1 shows 2 examples of ligand folding from Prone materials, from the extreme effect with the Kromasil C18 to partial collapse with the classic Zorbax ODS.

Ligand Folding Effects

Fig 1



EXPERIMENTAL

Using standard HPLC systems, the following operating conditions were used:

1. Columns: 150mm x 4.6mm size were used with 4 μ (Genesis) or 5 μ packing materials. They were packed in-house except where marked ** which were packed by the manufacturer.
2. Mobile Phase:- 0.1% aqueous phosphoric acid
3. Flow rate 1ml/min
4. Injection size, 10 μ l
5. UV detection at 210nm
6. Ambient temperature or 30°C (Jones Chromatography model 7971 heater/chiller) as stated
7. Column auto selector used for initial runs (Jones Chromatography series 7200)
8. Column regeneration typically used 95% acetonitrile/water (30minutes) via 5 column volumes of water.
9. Analyte mixture:- Urea (t₀ marker), formic acid, uracil, L-dopa, dopamine and gallic acid.

LIGAND FOLDING INFLUENCES

Bonded phases from several manufactures were tested for their propensity to ligand folding using a 6 component test mixture containing acidic, basic and neutral species. Not all of the phases were suitable for the analysis of the basic analyte, dopamine, but the final test probe, gallic acid, eluted from all the materials tested. The effects of various parameters were compared.

• General Retention Time Effects – Initial Study

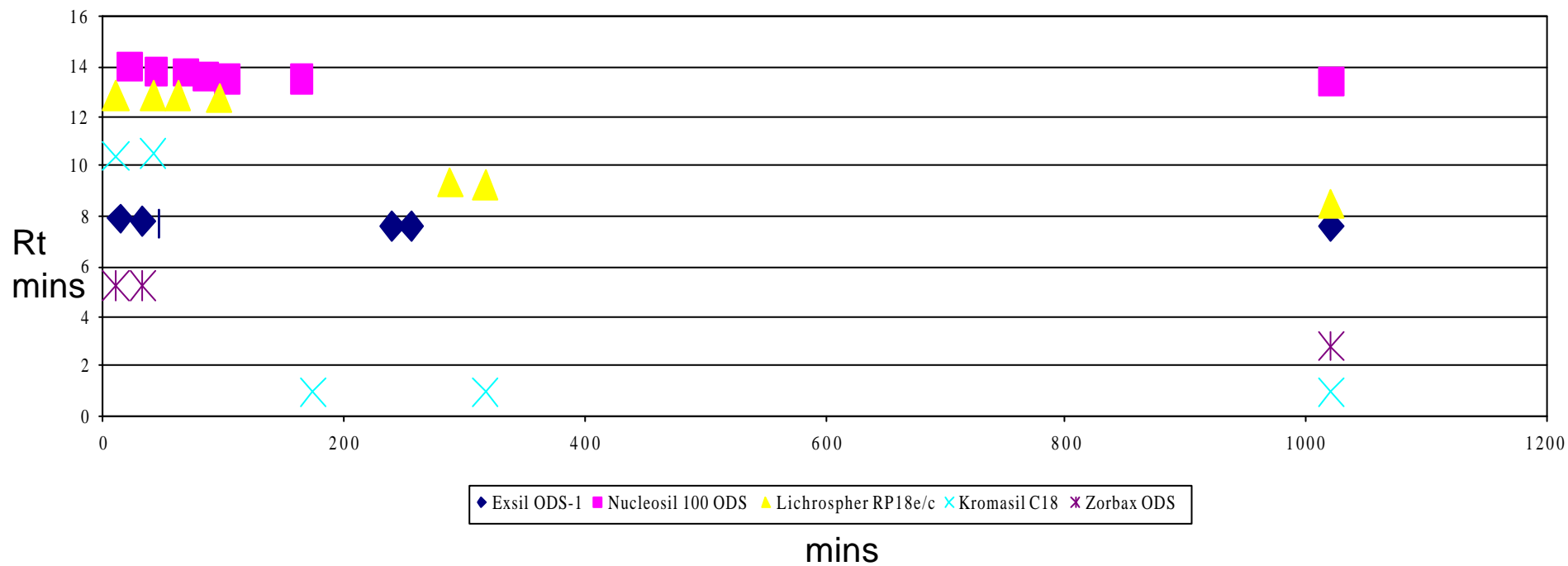
Using an automated column-switching device, at ambient temperature overnight, six columns were tested and the change of retention time plotted. Between injections the flow was stopped. The results show little change in performance indicating that the columns – Genesis AQ, Inertsil ODS-3, Genesis 120 C18, Genesis 300 C18 and Nucleosil 300 C18 are “inert” or “resistant”. In fact retention increased for some solutes and some columns, which is probably due to temperature effects. The change in retention over the 25 hour test period was -1.6 – +3.7%. Later work was therefore performed at 30°C.

• Effect of % Carbon and Surface Coverage Fig 2

From Fig 2 it can be seen that the high carbon and high coverage C18 materials such as Kromasil C18 (19% carbon, 3.1 μ moles/m²), classic Zorbax ODS (20% carbon, >3.7 μ moles/m²) and Lichrospher RP18e/c (21.5% carbon, >3.9 μ moles/m²), had a higher propensity to ligand folding. The lower coverage materials, such as Nucleosil 100 C18 (14% carbon, 2.1 μ moles/m²), have a lower ligand density and more space is available for the chains to bend without forming a matted surface. In the case of Exsil ODS-1 (7% carbon, 1.6 μ moles/m²), which is a partially bonded, mixed mode phase, the C18 groups have lots of room to move, the surface contains a high percentage of silanol groups and little ligand folding is observed. This is to be expected, the greater the ligand density the greater the hydrophobic nature of the surface and the greater ability of the chains to tangle and fold in on one another. The Kromasil showed one of the most marked collapse effects but does not appear to have the highest coverage and other phases of higher coverage were found to be much more resistant to folding. It is probable that the base silica characteristics and the ratio of the types of silanol present on the base silica play a role.

Effect of % Carbon and Surface Coverage

Fig 2



- **Effect of Pore Size Fig 3**

An increase in the pore size from 100-120A to 300A had little observed effect on the degree of ligand folding with the resistant Genesis C18. The Nucleosil 100 and 300A phases behaved similarly. The Nucleosil 100AB material, however, collapsed immediately indicating again that % carbon/surface coverage is very influential on phase collapse. However, from the column pressurization work done later, it is likely that larger pore materials are less prone to this effect and this would agree with theoretical concepts. See "Regeneration" later.

- **Effect of Bonded Phase Alkyl Ligand**

Two families of packing material were tested, Kromasil C18, C8, C4 (all end-capped) and C1 and Lichrospher RP18e/c and RP8e/c. They were chosen because the C18 materials showed marked ligand folding. The Kromasil C18, C8 and C4 collapsed immediately, i.e. within 10 minutes of being subjected to the purely aqueous mobile phase. As discussed earlier, the original Kromasil silica may well be influential in this collapse. The first Kromasil C18 column only showed partial collapse until the flow was stopped after which the ligands were completely folded. The C1 phase showed a slow loss of retention (8.8% over 88 minutes) whilst the column was in use but a large drop of 54% after standing in the mobile phase with no flow. However since C1 phases are known to degrade in high aqueous phases due to surface deactivation this may be responsible. The short TMS chains would find it difficult to fold in the accepted sense of the word.

The results for the Lichrospher RP18 and RP8 phases, both end-capped, indicated that the shorter C8 phase showed a reduced ligand folding effect which is to be expected from the relative sizes of the chains and their degree of hydrophobicity.

- **Effect of Polar Ligand**

A Genesis 120 CN phase used in the reversed phase mode was compared to the Genesis 120 C18. It was also found to be resistant /inert to ligand folding, the relatively short cyanopropyl chain is neither long enough nor hydrophobic enough to collapse. The change in retention time was 5.9% over a 94 hour period.

- **Effect of End-Capping Fig 4**

The effect of end capping was observed using Apex 1 ODS and Lichrospher RP18. The Lichrospher RP18 end-capped material showed a much greater ligand folding effect than the un-capped phase. This is to be expected, since the degree of ligand folding appears to be closely related to surface coverage, the greater the ligand density the greater the propensity of the ligands to fold to protect themselves from the aqueous mobile phase. In addition, the un-capped phase has an increased concentration of surface silanol groups which help to stabilise the phase under these high aqueous conditions.

In the case of the Apex, due to the lower surface coverage, both phases showed a low folding effect. This lower surface coverage of bonded phase is due to a lower silanol surface density on the base silica as determined by Si²⁹ NMR spectroscopy.

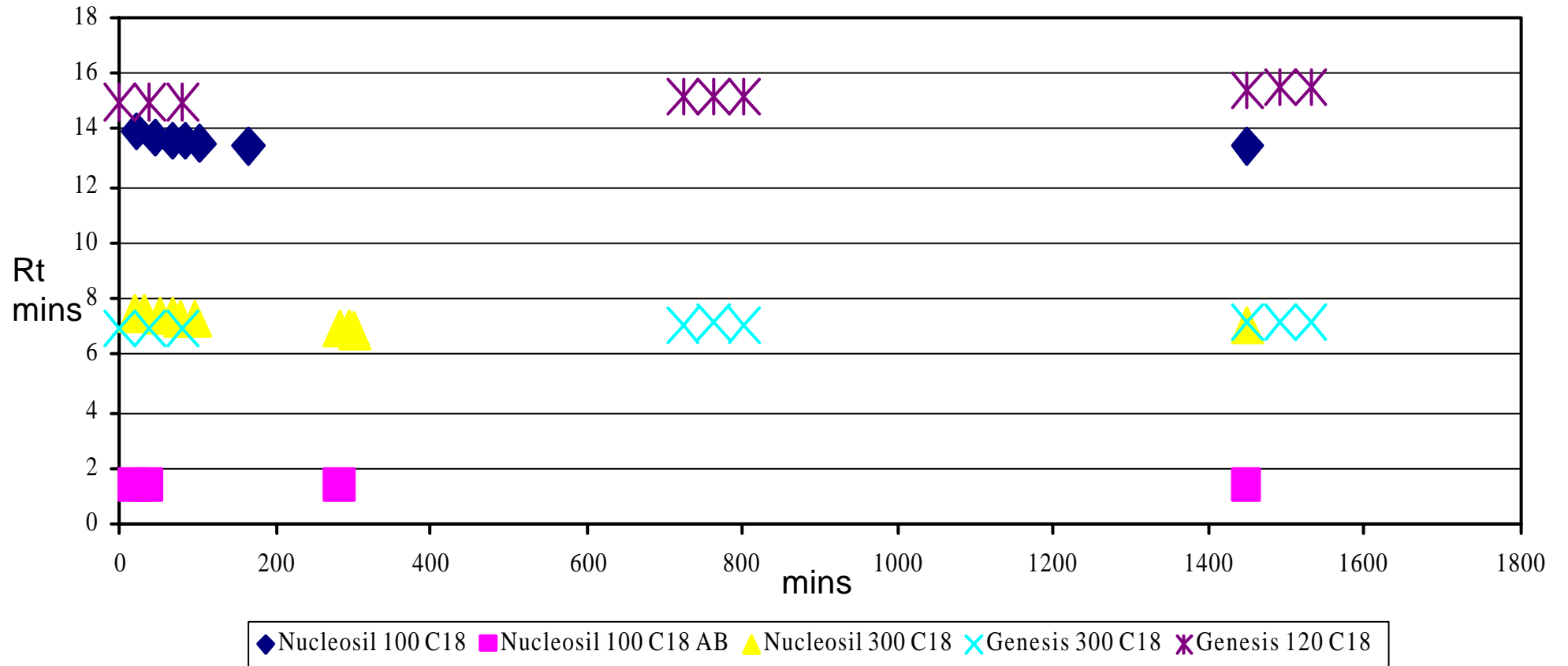
- **Effect of Ligand Collapse on Peak Shape and Efficiency**

Six columns, two from each category were compared from the viewpoint of column efficiency and peak shape. The flow was stopped between each injection time. The results are shown in the Table 2.

It can be seen that the prone phase lost column efficiency with time and in particular after each time the flow was stopped. Repeat injections around the same time frame showed very similar results. Peak shape was affected to a much smaller degree. There was no difference in this respect with the nature of the analytes tested.

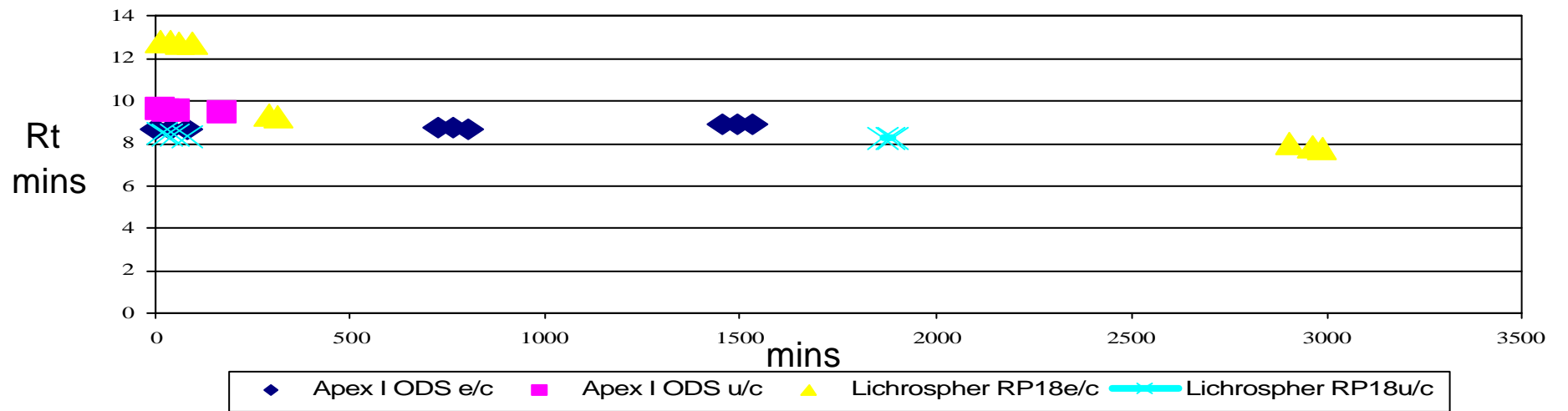
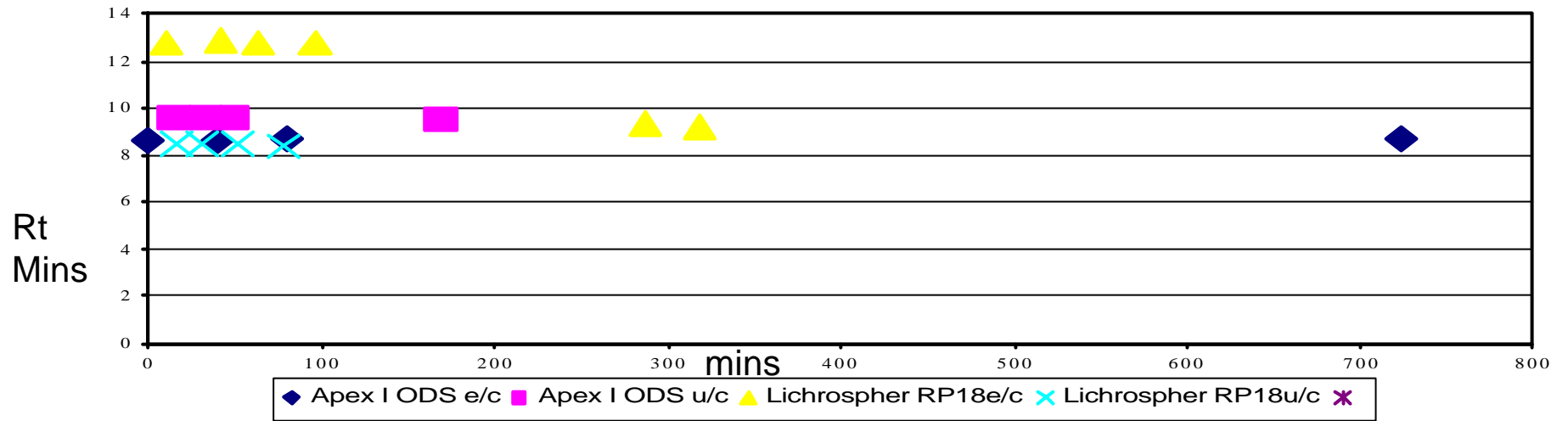
Effect of Pore Size

Fig 3



Effect of Capped and Un-Capped Phases

Fig 4



Peak Shape and Efficiency

Table 2

Material	Type	Time (min)	N dopamine	A _{10%} Dopamine	N gallic acid	A _{10%} gallic acid
Genesis AQ	Inert	40	79612	1.30	93507	1.20
Genesis AQ	Inert	764	77944	1.22	97312	1.22
Genesis AQ	Inert	1530	78324	1.22	95795	1.21
YMC ODS-AQ	Inert	38	59297	0.99	70395	0.91
YMC ODS-AQ	Inert	186	61243	1.09	68844	0.93
YMC ODS-AQ	Inert	1493	61772	1.01	70606	0.92
Genesis C18	Resistant	40	70248	1.26	82301	1.25
Genesis C18	Resistant	764	70469	1.22	83102	1.32
Genesis C18	Resistant	1530	67881	1.19	81915	1.31
Inertsil ODS-3 **	Resistant	40	41776	0.94	59572	1.07
Inertsil ODS-3 **	Resistant	764	41142	0.92	62082	0.85
Inertsil ODS-3 **	Resistant	1530	40653	0.88	59230	0.94
Lichrospher RP18e/c	Prone	40	42261	1.09	48743	0.95
Lichrospher RP18e/c	Prone	318	14569	1.17	20718	1.15
Lichrospher RP18e/c	Prone	2908	4027	1.32	5815	1.38
Genesis CN	Resistant	36	59318	0.89	50277	1.42
Genesis CN	Resistant	1554	60714	0.93	50893	1.45

- **Effect of Chemical nature of the Analyte**

The 6 component test mixture contained acidic, basic and amphoteric analytes but the ligand folding effect affected all the solutes in a broadly similar way.

- **Effect of Inertness and Material Purity**

No correlation was observed in the confines of this limited study, but the more recently introduced newer phases tested were resistant rather than Prone, despite a good surface coverage.

- **Equilibration Times**

Solvent regeneration times for re-use in purely aqueous mobile phases were determined. The resistant Genesis 120 C18 material was regenerated with 95% acetonitrile /water in less than 30 minutes whereas this time was insufficient for Kromasil C18 and Zorbax ODS. In the former case at least 100 minutes was needed and in the latter case 80 minutes was sufficient to re-instate the initial conditions. For re-use with 70% acetonitrile/ water mobile phase, an equilibration time of 15-20 minutes was sufficient for all phases. In all cases original column performance was restored.

REGENERATION

Columns that had been subjected to ligand collapse could be regenerated in two ways, either by flushing the column with a high organic mobile phase for a period of time, maybe up to over 100 minutes or by the application of high pressure to the column. It is desirable to use a mobile phase of 60-95% organic to reverse the folding process and then to re-equilibrate with the desired mobile phase if it is lower in organic content. This speeds up the regeneration process. This solvent method is the usual way of column regeneration. In one instance a column could not be reconditioned for further use with purely aqueous mobile phases although it was fine for high organic/ aqueous mixtures.

Several workers have observed the unfolding effect of high pressure on the column. An increase in pressure is generated within the column by fitting a fine capillary to the column outlet. In the experiments performed a fused silica capillary was threaded through a length of 1/16 PEEK tubing and inserted between the column outlet and the detector inlet. The pressure drop across the column is unchanged but the whole column is subjected to pressures up to 300 bar (4500psi). This will generate a pump pressure of around 400 bar (6000psi). This high pressure would appear to unfold the chains, speeding up equilibration and generating an increased alkyl surface for interaction with the solute molecules.

This is shown in Figs 5-6 for the Kromasil C18 phase, which exhibited total collapse of the chains and in the table below for a range of phases. For the Kromasil column the standard pressure drop across the column was 49 bar. With the application of the 17cm capillary the pump pressure increased to 120 bar and a slight increase in retention is observed. When the longer 30cm capillary was applied, pump pressure increased to 230 bar, giving a column outlet pressure of 181 bar and a long retention time with full peak resolution is observed. This retention was much greater than the previous "initial" value for the column. The use of a 40cm capillary restrictor increased excess pressure to 350 bar and increased the retention to over 20 minutes. It is difficult to give an "initial" value since some time for equilibration must be given but the folding will start as soon as the alkyl chain environment becomes highly aqueous. By pressurising the column a theoretical initial value can be obtained, the pressure needing to be increased until a maximum retention is reached. However a pressure greater than the maximum of the

analytical pump may be required. In fig 6 the pressure restrictor was removed and a rapid reduction in retention was observed. However, good resolution was still observed and reproducible retention was seen for 3 successive injections over a period of more than 1 hour. However, switching off the flow and allowing the column to depressurise for 30 minutes was enough to completely collapse the phase again.

For Kromasil C18

Stage	Retn.Time, gallic acid	N/m, gallic acid	A10%, gallic acid
Initial, 49 bar (no restrictor)	1.30	N/A	N/A
H.P.Regeneration, 120bar	1.62	N/A	N/A
H.P.Regeneration, 230 bar	15.85	75083	1.13
49 bar, run 1	4.93	35510	1.21
49 bar, run 2	4.92	36216	1.20
49 bar, run 3	4.91	35071	1.23
Stop/Start, run 4	1.30	N/A	N/A

Application of pressure affected other phases as follows:-

Phase	Pressure across Column	Retention Time, Before, mins	Retention Time, After, mins	% Rt Change
Genesis AQ	198 bar	14.46	15.20	5.1
YMC ODS-AQ	205 bar	14.37	15.36	6.9
Genesis C18	211 bar	13.29	14.91	12.2
Kromasil C18	230 bar	1.30	15.85	1119.2
Lichrospher RP18	203 bar	13.27	20.84	28.1
Apex 1 C18	207 bar	9.83	10.77	9.2
Zorbax SB300 C18	202 bar	3.57	3.71	3.9
Genesis CN	190 bar	6.24	6.27	0.5

The inert AQ phases showed a small increase in retention indicating marginal folding and the resistant C18 a slightly increased folding effect. The 300A Zorbax 300 SB C18 and the polar Genesis CN showed a small retention time change indicating minimal folding. This can be used as a test for ligand folding. It is probable that a combination of pressure and organic solvent would be the fastest way of regenerating the column.

An alternative way of looking at this chromatographic affect is in terms of wettability. Because of the hydrophobic nature of the surface, especially where the ligand density is high, water cannot enter the pores and so the amount of ligand surface area capable of interacting with the solute is very small – potentially only the surface area of a 4 μ or 5 μ sphere, the majority of surface area being within the pore region. The effect of pressure is to force the aqueous mobile phase containing the analytes into the pores of the particle (cf. mercury porosimetry, used to determine pore size distributions in silicas). The elution time for the first peak with the Kromasil C18 under pressurized conditions was 1.41 minutes compared to a value of 1.02 minutes at atmospheric column outlet pressure. Using a 5 million molecular weight standard and THF

solvent with this column, the excluded column value was found to be 0.86 minutes. The higher value of 1.02 minutes above is not unexpected since the top of the column is under slight pressure and any larger pores present will have a reduced wettability affect. Although we have yet to test this experimentally, the content of the pore may be a water/acetonitrile mixture equal in composition to that which will just wet the surface. If any analyte did manage to penetrate the pore region, the relatively high organic level would result in negligible retention in the stationary phase. The pore size and size distribution would play a part in the wettability effect but only large changes would be expected to be chromatographically noticeable. The pressure required to restore the peak resolution would depend on the pore size and size distribution of the packing, the smaller the pore the greater the pressure needed ($P = 2\sigma \cdot \cos\theta / r_p$ where P = pressure, σ = surface tension, θ = contact angle and r_p = pore radius)).

The use of high organic solvent mixtures also overcomes the wettability problem, the stationary phase surface becomes rewetted and allows mobile phase and analytes to enter the pores. Their surface tensions are lower and the surface contact angles are lower ($<90^\circ$).

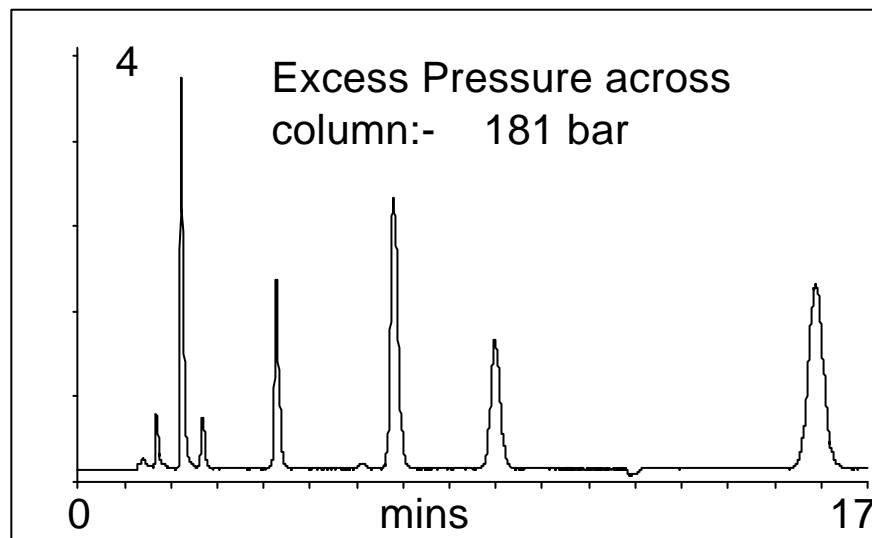
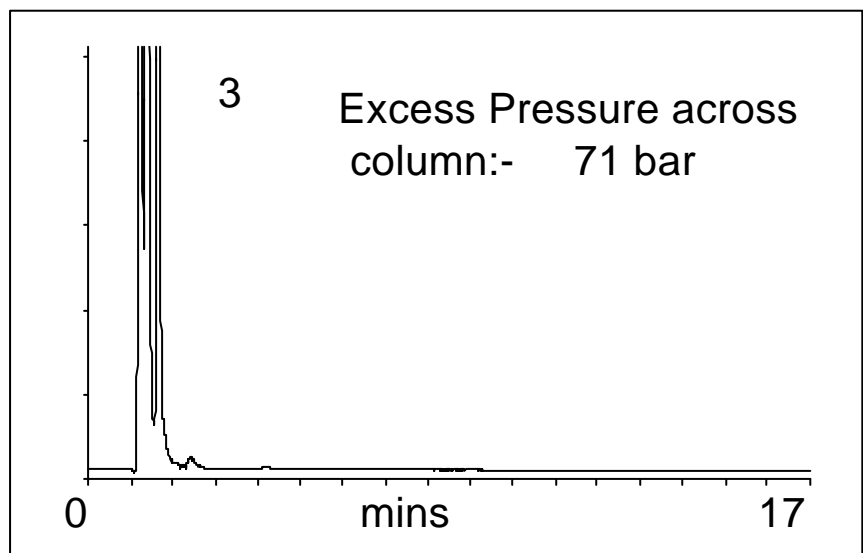
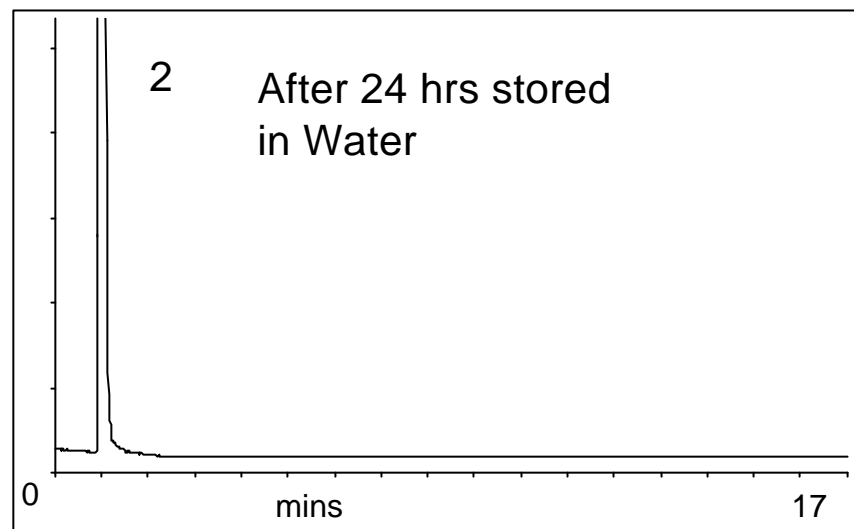
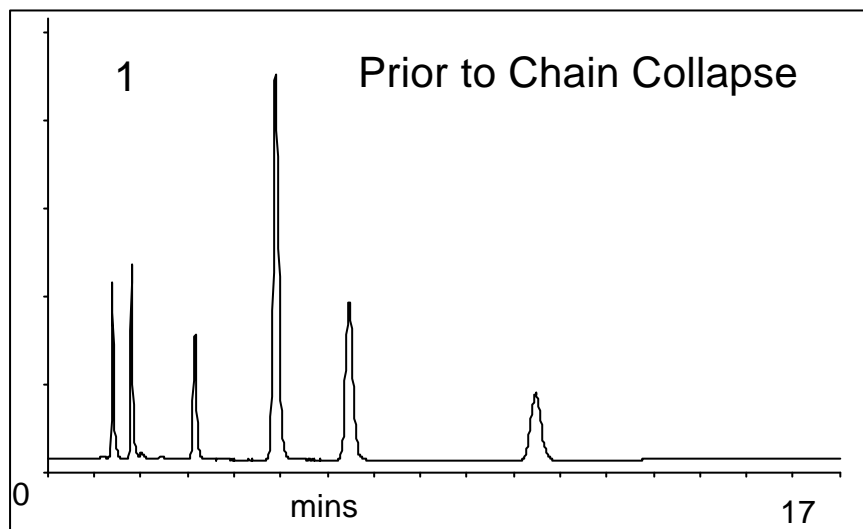
COMPARISON of AQUEOUS and STANDARD C18 COLUMNS

Comparing the “inert”, “high aqueous” Genesis AQ and the “resistant” Genesis 120 C18, with non-polar and polar analytes in high organic mobile phases, we observed that the Genesis 120 C18 showed an increased retention for non-polar analytes compared to the AQ phase but reduced retention for polar and moderately polar analytes. Three test mixtures were chosen, the first being a selection of poly-aromatic hydrocarbons run with 85% methanol/ water where all analytes were more retentive on the C18 column, pyrene having retention times of 5.72minutes compared to 5.00 minutes on the AQ. The second mixture comprised 5 carboxylic acids from formic to propionic run in 2% acetonitrile/ 0.1% phosphoric acid. Retention times for the final peak, propionic acid was 2.75 minutes for the C18 compared to 3.65 minutes for the AQ. The third test mix, a conventional test mixture run with 70% methanol/water gave retention times for phenol and toluene for the C18 column of 0.46 and 3.39 minutes respectively and for the AQ column the corresponding values were 0.51 and 3.31 minutes. The latter chromatogram is shown in fig. 7.

This is to be expected for the non-polar analytes since the Genesis 120 C18 has an 18% carbon loading compared to the 15% for the AQ phase, both materials having the same base silica. The coverage of the materials is similar at $3.6/3.7\mu\text{moles}/\text{m}^2$, coverage being calculated from the sum of the individual bonded ligand types at the surface. For the polar molecules, the special bonding of the AQ preserves hydrophilic sites at the silica surface whilst maintaining deactivation and this leads to an increase in retention for polar molecules. Thus selectivity differences occur between the “high aqueous” and conventional C18 alkyl phases.

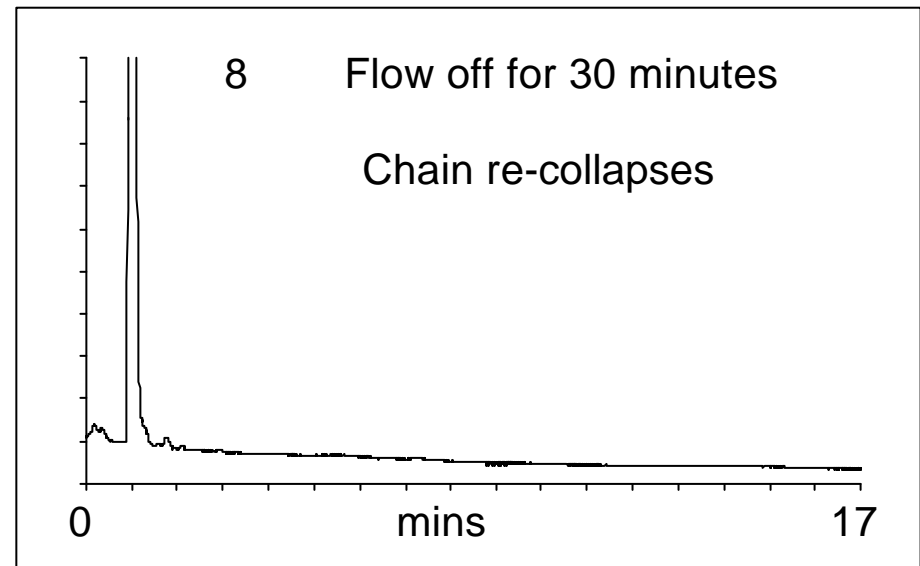
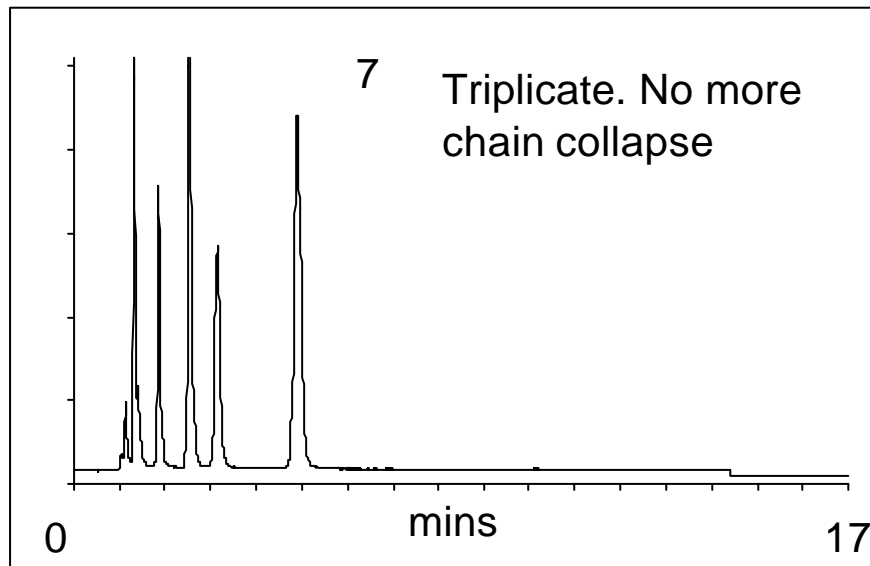
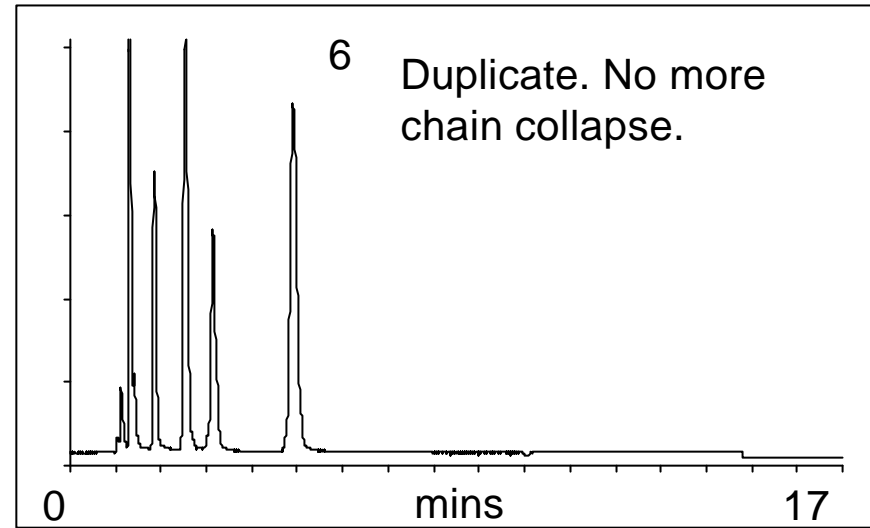
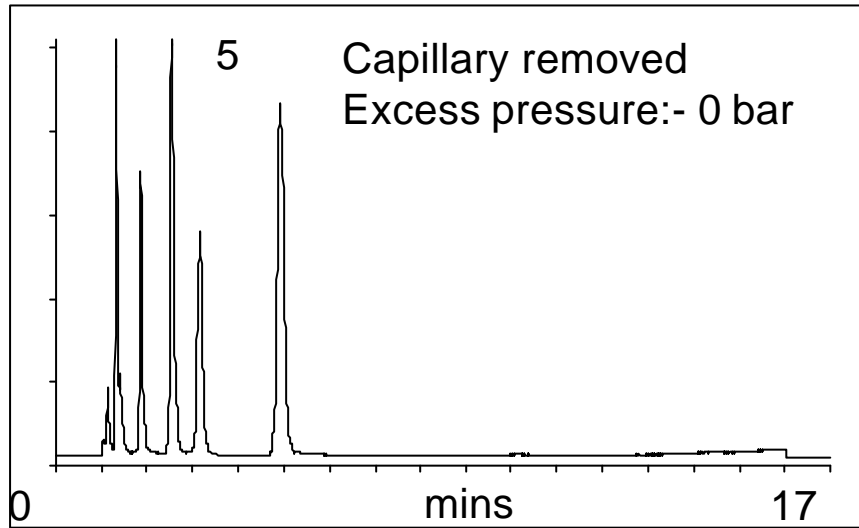
Fig 5

Pressure Regeneration 1



Pressure Regeneration 2

Fig 6

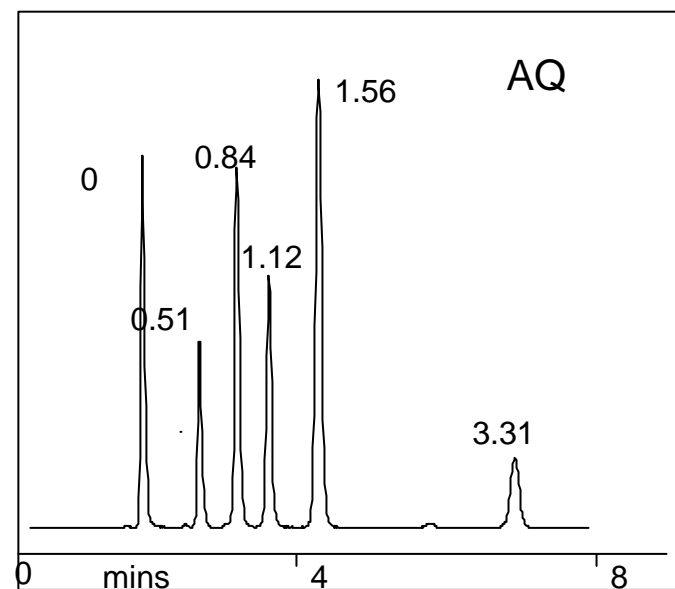
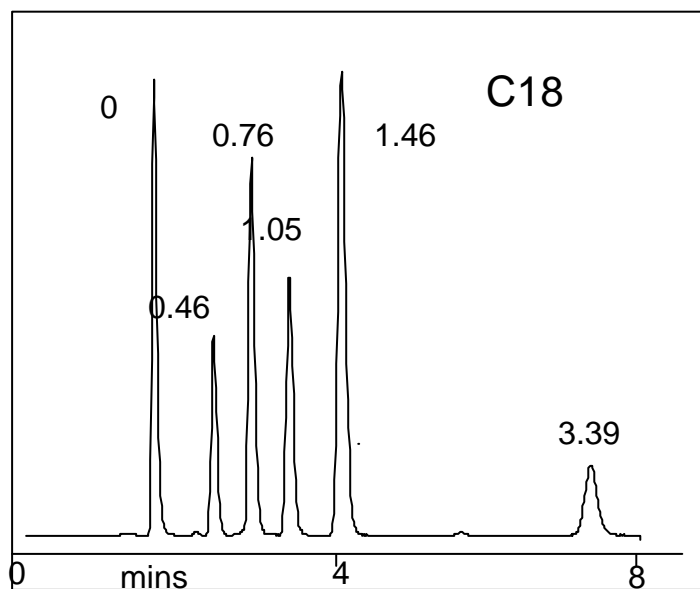


C18 vs. AQ Comparison - Mixed Polarity

Fig 7

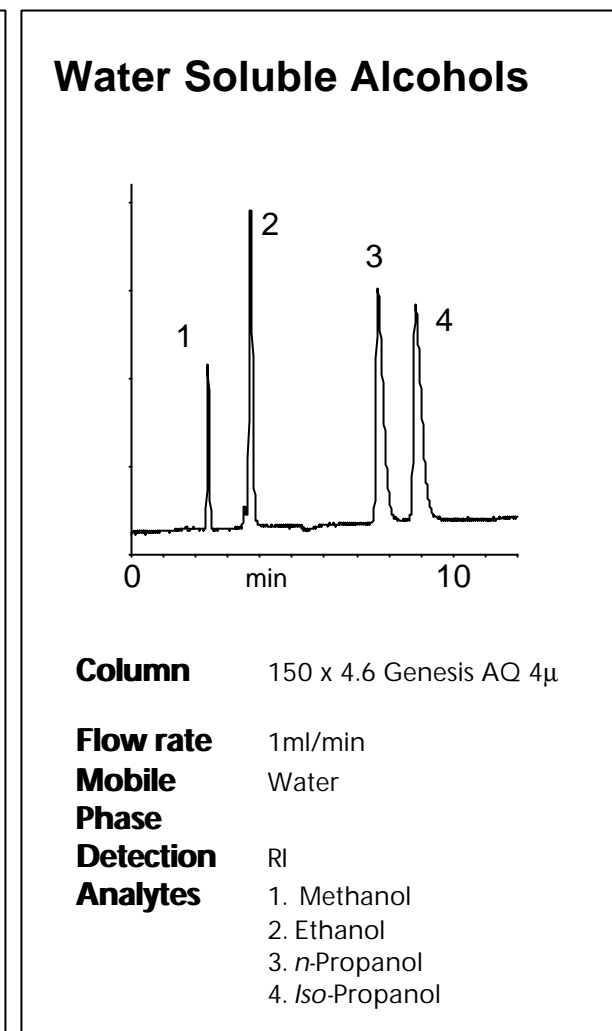
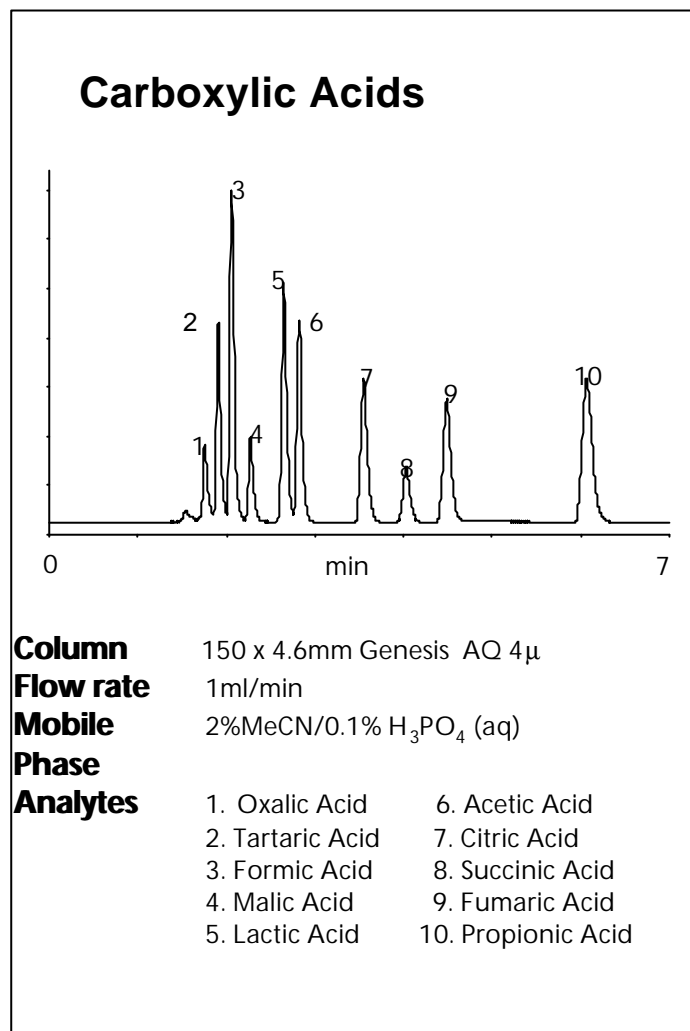
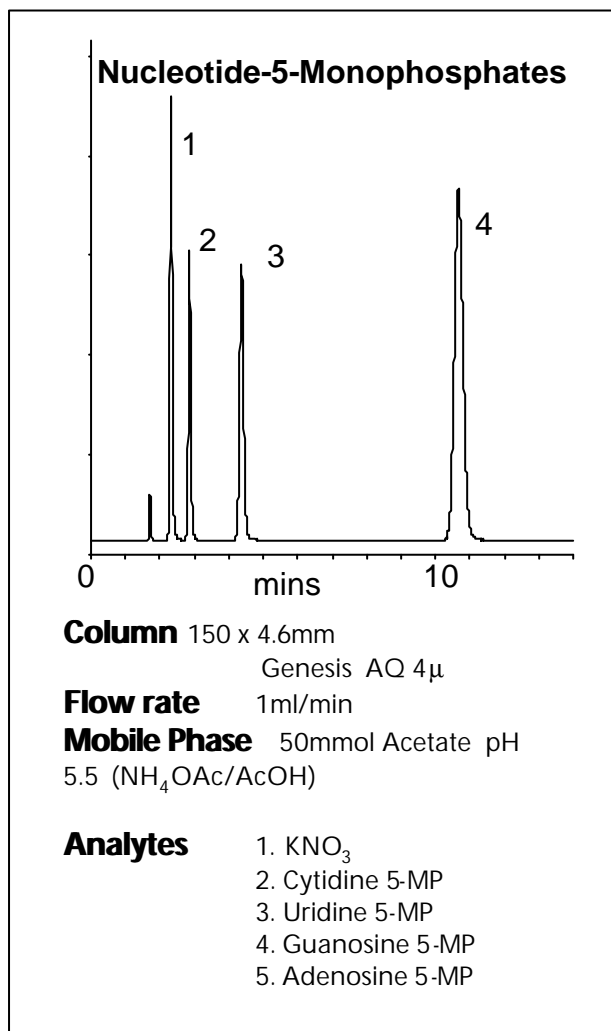
Column:- 150 x 4.6mm Genesis, as shown
Mobile Phase:- 70% Methanol / water
Flow Rate:- 1ml/min
Analytes:- Uracil, phenol, acetophenone, nitrobenzene,
methyl benzoate and toluene

k' values



APPLICATIONS

Application areas for “high aqueous” columns are polar, hydrophilic compounds, usually water soluble. Three examples are given below.



CONCLUSIONS

- Stopping flow has a marked effect on retention change due to its effect on ligand folding. Pressure in the active column acts to prevent total collapse.
- Percent carbon and surface coverage have the most influence on the degree to which ligand folding occurs, the higher the values the more likely the phase will undergo collapse.
- End-capped phases are more prone to ligand folding than uncapped phases.
- Although shorter chains in general are likely to be less prone to this effect, the type of base silica used is also important.
- The AQ type phases showed an increased retention for polar analytes and a lower retention for non-polar analytes when compared to a similar C18.
- Although the effects are normally reversible, phases very prone to ligand collapse may be difficult to recondition for use in high aqueous modes but they are still satisfactory for use with higher levels of organic modifier.
- The chromatographic effects can be explained in terms of phase wettability. Column pressure can play an important role in reversing the process by forcing solvent into the pores of the packing.
- Ligand folding is a function of the material and should not be construed as a negative effect except in terms of this type of application. Invariably the material was designed for other applications where it no doubt performs well.