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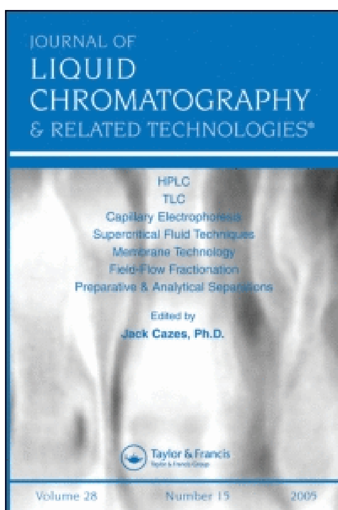

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High Performance Liquid Chromatographic Analysis of Sulphonamides and Dihydrofolate Reductase Inhibitors. III. The Effect of a Competing Base, and Separations with an Ion Pairing Agent

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SULPHONAMIDES AND DIHYDROFOLATE REDUCTASE INHIBITORS. III. THE EFFECT OF A COMPETING BASE, AND SEPARATIONS WITH AN ION PAIRING AGENT

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ABSTRACT

The effect of tertiary butyl ammonium phosphate as a competing base has been investigated in the reverse phase separation of twenty-two sulphonamides (SFA) and three commonly used dihydrofolate reductase inhibitors (DHFR). At the concentrations of t-butyl ammonium phosphate examined, the retention of the DHFR was dramatically reduced, but did not aid the separation. The effects on the SFA were inconsistent with the known $pK_{a,1}$ data and suggested either more complex mechanisms of interaction with the stationary phase or some doubt regarding the $pK_{a,1}$ values.

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Heptane sulphonic acid (HPSA) was tested as an ion pairing agent. Large effects were observed for the three DHFR but only a few of the SFA. Again, the results were inconsistent with the known $pK_{a,1}$ data. Low concentrations of HPSA were found to be most useful, and slightly different concentrations had significantly different effects in several parts of the chromatogram. Thus, 0.001 M phosphate buffers around pH 2.9 were modified with 0.5 and 1.0 mM HPSA and combined with previously determined MEOH gradients. Sulphamoxole and sulphamethizole were inseparable, but all other pairs of compounds had $R_s \geq 0.9$. The result is clearly superior to any previous HPLC separation .

INTRODUCTION

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In previous papers on the reverse phase retention behaviour of twenty-two sulphonamides(SFA) and the three commonly used dihydrofolate reductase inhibitors(DHFR), we have demonstrated[1,2] that most combinations of the drugs are separable. In the context of a total separation of the 25 drugs in a reasonable analysis time, there are two commonly recurring difficulties. Sulphathiazole(ST, usually eluting as the eighth sulphonamide) and sulpha-pyridine(SP, 9) were frequently not separated. The other group of drugs that were difficult to separate under the majority of conditions investigated, were sulphameter(SM, 11), sulphamoxole(SAM, 12), sulphamethazine(SMAZ, 13), diaveridine(DVD, 14) and sulphamethizole(SMIZ, 15). These five compounds were generally tightly bunched, usually included more than one coelution and often overlapped with the next compounds to elute.

In methanol modified mobile phases[1], the optimum pH[3,4] was confirmed to lie between 2.7 and 3. Twenty peaks were commonly discernable, and in the best case, all but two compounds could be distinguished. SAM(12) and SMAZ(13) were coincident, and, ST(8) and SP(9) were only partly resolved. However, the other 21 drugs and the commonly observed hydrolysis product (of SAM) and the one usually hidden were both clearly resolved. Although no results were shown for acetate, separations in these buffers were inferior to those in phosphate. For multiple drug analyses, there were several generalisations that could be made. Firstly, at low phosphate concentrations (0.001 M) the DHFR are eluted much earlier. This removes DVD from the crowded central section of the chromatogram (although there is still one multiple elution in the middle). With an appropriate gradient, the last 10 drugs elute almost perfectly. On the other hand, the front of the chromatogram is not as good under these conditions. Secondly, at higher phosphate concentrations (0.01-0.1 M), the front end of the chromatogram is improved at the expense of the back end. ST and SP show some degree of separation. Thirdly, higher pHs

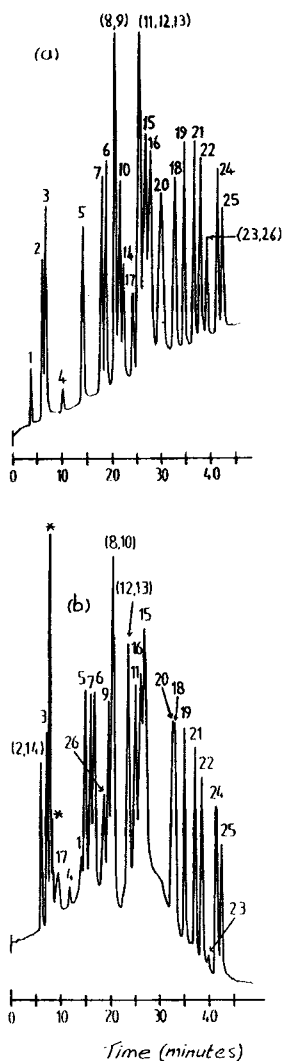

Figure 1. Complex ternary gradient and flow programmed elution (see Table 1) at pH 2.80. Chromatogram (a) was obtained without TBA and (b) was obtained with 0.1 g/L of TBA added to the aqueous buffer. The compounds are: **(1)**SNAC, **(2)**SG, **(3)**SAN, **(4)**SAM hydrolysis product, **(5)**SAC, **(6)**SDZ, **(7)**SISM, **(8)**ST, **(9)**SP, **(10)**SMRZ, **(11)**SM, **(12)**SAM, **(13)**SMAZ, **(14)**DVD, **(15)**SMIZ, **(16)**SMP, **(17)**TMP, **(18)**SCP, **(19)**SMOX, **(20)**SST, **(21)**SISX, **(22)**SB, **(23)**PST, **(24)**SDIM, **(25)**SQ, **(26)**PYR.

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are generally unfavourable and lead to excessively congested chromatograms. However, there is one exception. Due to fortuitous differences in the pK_a[2] values, the most difficult to separate group (11-15) are baseline resolved from each other at pH 6.5.

In acetonitrile modified solutions,[2] separations are not quite as good, but some minor selectivity differences do occur. Attempts to incorporate the beneficial differences into MEOH gradients were unsuccessful, apparently due to complex chromatographic behaviour. Returning to MEOH modified mobile phases, flow programming was successful in the resolution of the first 13 compounds, including the seldom separated ST and SP ($R_s \approx 1.1$)[5].

Separation of all 25 drugs simultaneously has not been achieved (Fig. 1). Hence, we have investigated other solution variables. Although the stationary phase was end-capped and the peaks did not show excessive tailing, the effect of a competing base is sometimes unpredictable, such that advantageous selectivities may arise. Hence, in this paper we examine this effect, and finally, ion pairing.

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EXPERIMENTAL

With the exception of the laboratory reagent tetrabutyl ammomium phosphate (TBA; Sigma, St. Louis, MO) and the reagent grade heptane sulphonic acid (HPSA; Aldrich, Milwaukee, WI), all chemicals, equipment and experimental methods were as previously described.[1,2] The pH was set at 2.80 (unless otherwise specified) and 0.001 M phosphate buffer was used in all experiments. In the case of TBA (0.1 g/L) and HPSA, the additional component was added to the aqueous buffer before pH adjustment. The column oven was set to 33 Celcius for all experiments.

The full names of the DHFR, their abbreviations and pK_a values are given in the results and discussion section where they are required and the equivalent data for the SFA is given in Table 2. The structures of all of the drugs have been listed previously.[6]

RESULTS AND DISCUSSION

The Effect of a Competing Base

The gradient used to examine the effect is given in Table 1. It is a complex ternary gradient involving both MEOH and ACN, and flow programming. Its choice is not significant. It happened to be the last of such

Table 1

Gradient Program Used for the Chromatograms Shown in Figure 1

Time (Minutes)	% 0.001 M Phosphate	% MeOH	% ACN	Flow Rate (mL/Min)
0	100	0	0	1.0
5	95	0	5	0.7
9.5	87	5	8	1.0
15	81	8	11	1.0
20	75	10	15	0.8
25	73	11	16	0.6
30	71	12	17	0.2
35	67	15	18	1.0
45	76	0	24	1.0
50	70	0	30	1.0

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runs done at the time. Figure 1(a) is the reference chromatogram and Figure 1(b) shows the result of the addition of 0.1 g/L of TBA to the aqueous phase. The peaks marked with an asterisk in Figure 1(b) are impurities in the TBA.

In spite of using an end-capped stationary phase with good peak symmmetry indicating minimal evidence of mixed retention mechanisms, the retention times of the DHFR are dramatically reduced in the presence of the competing base. Taking sulphanilic acid (SA, 1) as an indication of t_m (Figure 1(a), ~4 minutes), the amount of retention (t_R') is reduced from 18 to 2 minutes for diaveridine (DVD, compound number 14), 20 to 5½ minutes for trimethoprim (TMP, 17) and 35 to 14½ minutes for pyrimethamine (PYR, 26). This clearly indicates that the principle retention mechanism for these drugs involves interaction with the surface silanols. As the three DHFR are all 2,4-diaminopyrimidines it would be expected that their ion exchange, ion-dipole and/or dipole-dipole interactions with silanol groups would be similar. The reductions in retention caused by the addition of the TBA are of similar magnitudes (16, 14½ and 20½ minutes) as expected. With $pK_{a,1}$ values close to 1.3,[7] $pK_{a,2}$ = 6.6 and 7.0 for TMP and PYR, respectively, and each pK_a refering to a deprotonation of a pyrimidine ring nitrogen, at the mobile phase pH of 2.80, the DHFR carry an average charge around +1.03. Hence, ion exchange is possible at underivatised surface silanol sites. This is consistent with the possible 'adsorption' of the DHFR on the walls of untreated silica capillaries in CZE (at pH 2.1)[7], and the adsorption of other cationic species on silica at low pH[8,9]. A full discussion of this and alternative possible interactions was given.[7]

Table 2

Changes in Retention for the Sulphonamides in the Presence of TBA

Sulphonamide (Abbreviation)	Number	Change in Retention (minutes)	$pK_{a,1}$	$pK_{a,2}$	Charge at pH 2.80
Sulphamethoxypryridazine (SMP)	16	-2	n/a	6.7	---
Sulphadiazine (SDZ)	6	-2	2.0	6.5	+.14
Sulphisomidine (SISM)	7	-2	n/a	n/a	---
Sulphamoxole (SAM)	12	-1½	n/a	n/a	---
Sulphamethazine (SMAZ)	13	-1½	2.4	7.4	+.28
Sulphapyridine (SP)	9	-1	2.6	8.4	+.39
Sulphamerazine (SMRZ)	10	-1	2.3	7.0	+.24
Sulphameter (SM)	11	-½	n/a	6.8	---
Sulphaguanidine (SG)	2	0	n/a	11.3	---
Sulphanilamide (SAN)	3	0	2.4	10.4	+.28
Sulphathiazole (ST)	8	0	n/a	7.2	---
Sulphamethizole (SMIZ)	15	0	n/a	5.4	---
Sulphachloropyridazine (SCP)	18	0	n/a	5.5	---
Sulphamethoxazole (SMOX)	19	0	n/a	5.6	---
Sulphisoxazole (SISX)	21	0	1.5	5.1	+.05
Sulphabenzamide (SB)	22	0	1.8	4.6	+.09, -.02
Phytalyl sulphathiazole (PST)	23	0	2.9 (ca)	7.2 (ca)	-.44
Sulphadimethoxine (SDIM)	24	0	n/a	6.2	---
Sulphaquinoxaline (SQ)	25	0	n/a	5.5	---
Sulphacetamide (SAC)	5	+1	1.8	5.4	+.09
SAM hydrolysis product (HP)	4	+1½	n/a	n/a	---
Succinyl sulphathiazole (SST)	20	+2	4.2 (ca)	7.2 (ca)	-.04
Sulphanilic acid (SNAC)	1	+10	n/a	3.2	-1, +.7

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Some of the cationic SFA also display reduced retention in the presence of the TBA. Table 2 (column 3) lists the SFA in order from the greatest reduction in retention to the least. All of the losses in retention are small, and where pK_a data is available, it is clear that the compounds experiencing reduced retention due to the presence of TBA are all partly in the form of the positively charged conjugate acid. However, there appears not to be any correlation between the exact magnitudes of the losses of retention and the average charges (last column). Furthermore, SAN has the same $pK_{a,1}$ (and therefore positive charge) as SMAZ but is unaffected by the TBA presence. The effect of the competing base is certainly a marginal effect on the SFA, with the majority having exactly the same retention times in the presence of TBA as they had in its absence. With the exception of SNAC, SST, HP and PST, all of the SFA are 4-aminobenzenesolphonamides and at low pH are partly protonated on the 4-amino substituent. As this protonation is so far removed from the point of substitution on the amide that distinguishes the molecules, it is not suprising that

the $pK_{a,1}$ values fall in such a small band, and that the size of the effect of substitution is not highly variable. (Those SFA for which a $pK_{a,1}$ is not available (n/a) would be expected to have values in the same range.) With the exception of SAN, it appears that the more positively charged SFA are affected by the presence of TBA, and by implication undergo small, but significant, interactions with surface silanols, whilst those with smaller positive charges do not (SISX, SB and SAC). This indicates that the nature of the SFA-silanol interaction may be ion exchange.

At the bottom of the third column of Table 2 there are four SFA which experience increases in retention in the presence of TBA. This is presumed to be due to 'ion pairing' and is an effect often observed in the presence of competing bases.[10] For this to happen, a negative charge is required on the analyte. SNAC, which is a relatively strong acid, exists as a zwitterion below the pH range for the deprotonation of the positively charged 4-amino group ($pK_{a,2}$ = 3.2).[11] Thus, at pH 2.8, SNAC is 30% anionic and retention is dramatically increased from ~0 to 10 minutes. SST is only 4% anionic and the increase in retention is only 2 minutes. PST is the other SFA which would be expected to 'ion pair' with TBA. No effect is observed. It should be noted that, for SST and PST the $pK_{a,1}$ and $pK_{a,2}$ values are assumed to be the same as for the component parts and the values relevent to the calculation of the charge at pH 2.8 ($pK_{a,1}$) are most likely to be different. From the pH dependence of retention in methanolic mobile phases[1], the observed inflection points indicate $pK_{a,1}$ values of ~4.5 for SST and ~3.75 for PST. These values would greatly decrease the negative charges on both of these SFA, but do not rectify the inverse correlation between charge and the extent of increased retention for SST and PST. Worse still, the revised $pK_{a,2}$ value of ~4.5 for SST would reduce the charge to -.02, in which case any significant 'ion pairing' might be suprising.

Overall then, whilst it does seem clear that TBA exerts the conventional effect of a competing base and negatively charged analytes may be 'ion paired' with it, the lack of quantitative correlation in each case and the exceptions, indicate that the retention mechanisms and perhaps their mutual modification may be much more complex. These factors also bring into question the very basis for the above analysis, which was, that the identical retention times for half of the SFA in the absence and presence of TBA could be assumed to be due to the absence of both of these effects. Perhaps it is a balancing of multiple, more complex retention mechanisms that leads to the zero net effects and specific molecular characteristics that lead to the unexpected imbalances that provide the exceptions.

In terms of the attempted separation, there were three coelutions containing seven compounds in the absence of TBA, whereas there were four coelutions containing eight compounds in the presence of TBA. The separation

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Table 3

Ternary Gradient Used to Generate the Data Shown in Figures 2 and 3 The Ion Pairing Agent Heptane Sulphonate was Included in the Aqueous Phase.

Time (minutes)	% 0.001 M Phosphate	% MeOH	% ACN
0	100	0	0
0.01	95	5	0
7	92	8	0
7.01	96	0	4
21	96	0	4
26	84	16	0
31	82	18	0
36	70	30	0
60	70	30	0
60.01	50	0	50
90	50	0	50
110	0	0	100

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of compound 11 from 12 & 13 and the removal of SNAC(1) from the vicinity of the solvent front were advantages obtained by the inclusion of TBA in the mobile phase. Overall, there was little net gain, but the highly selective nature of the effects of adding the competing base make it a potentially useful adjunct to separation in appropriate circumstances.

Ion Pairing

Ion pairing (IP) is the term used to describe enhanced retention as the result of the addition to the mobile phase of a large ion of opposite charge to the molecular ions to be separated. The ion pairing agent (IPA) used is generally a bulky, buried charge alkyl ammonium ion of the general form $R_1R_2R_3R_4N^+$ for the increased retention of molecular anions. TBA is typical.

For molecular cations, a dispersed charge anion with a non polar tail such as alkyl sulphates or sulphonates are generally utilised. In this study, heptane sulphonate (HPS^-) was used. These IPA are chosen because they have a great deal of non polar character and will be trapped in equilibrium concentrations in association with the non polar stationary phase. Due to the requirement for electroneutrality, the HPS^- counter ions will also be present in the modified

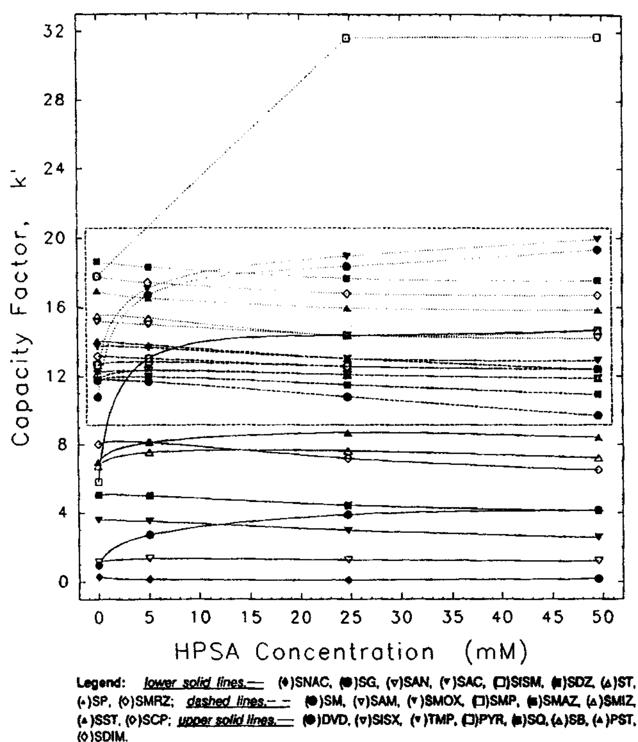

Figure 2. Plots of k' vs the concentration of HPSA for the 22 SFA and 3 DHFR compounds. The gradient used is given in Table 3 and the pH was 2.80.

mobile phase making the process of retention enhancement more one of ion exchange than IP. However, that is the common terminology and we will use it without further qualification.

Table 3 is the (ternary) gradient used for the initial examination of the effect of the IPA. HPSA was added to the aqueous buffer phase (only) prior to pH adjustment. Figure 2 shows the variation of the capacity factor, k', versus the concentration of HPSA. The crowded (boxed) central section is enlarged in Figure 3. Again, the data are not straight forward. SNAC, SST and PST have net negative charges (Table 2) and would therefore be expected to experience some repulsion from the stationary phase modified by the presence of HPS^-. All are progressively less well retained as the $[HPS^-]$ increases, but PST and SST display very similar behaviour in spite of appearing to have very different magnitudes of charge. Moreover, most of the SFA exhibit the same decreases in

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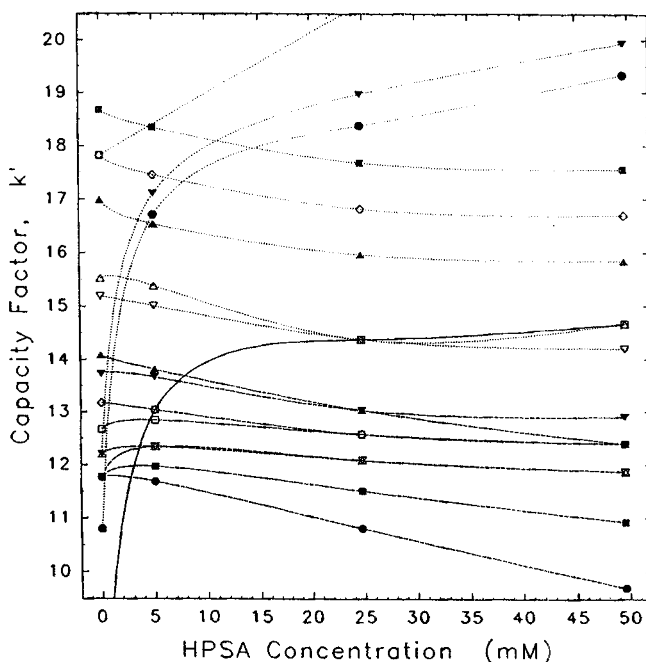

Figure 3. Enlargement of the boxed area in Figure 2 showing plots of k' vs the concentration of HPSA for the SFA and DHFR. All conditions and legends are as in Figure 2.

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retention as [HPS⁻] increases in spite of their positive charges (see Table 3 and the discussion in the previous section). We are unable to explain these results. Large increases in retention of the DHFR are observed as [HPS⁻] increases, as expected. A proportionally larger increase occurs for SISM, but unfortunately there is no $pK_{a,1}$ data in this case. A high value is indicated. This is also true for SG. SP with the largest $pK_{a,1}$ value and positive charge (of the SFA) has increased retention, but SMAZ and SMRZ - with similar charges - do not.

With several compounds displaying each different type of observed behaviour, there are multiple crossovers in the $k^{/}$ vs. [HPSA] plot. As was noted in the ACN work[2], this provides good separations of some pairs that have been hitherto difficult or impossible under most or all circumstances (for example, SG and SAN, and, SP and ST); but it also provides a multitude of new coelutions and the fusion of several $k^{/}$ vs. [HPSA] plots.

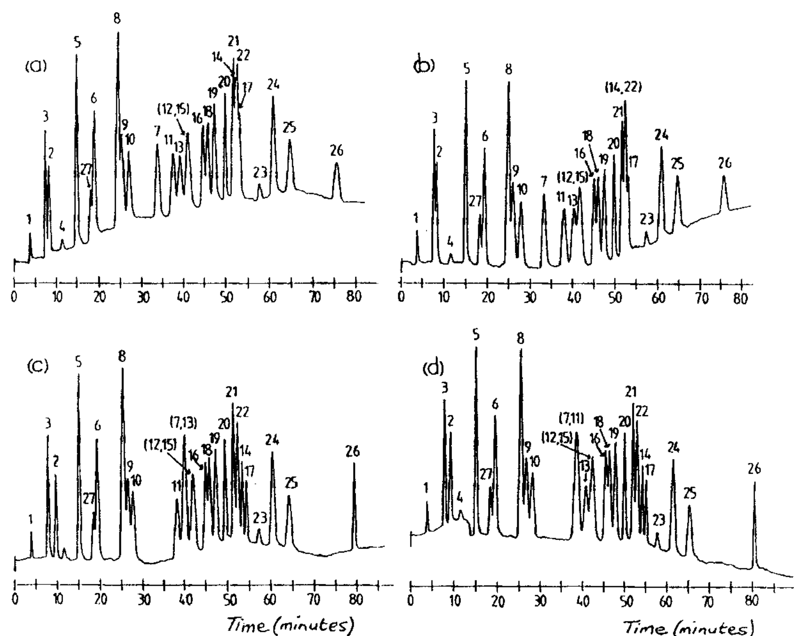

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Figure 4. Chromatograms obtained using the binary MEOH gradient in Table 4. The aqueous buffers were (a) pH 2.86 and (b) pH 2.97, and were both 0.5 mM with respect to HPSA. (c) pH 2.85 and (d) pH 2.96 were 1.0 mM with respect to HPSA. The compounds are numbered as in Figure 1. **27** is the second SAM hydrolysis product.

Table 4

Binary Gradient Used to Generate the Chromatograms Shown in Figures 4(a) - (d).

Time (minutes)	% 0.001 M Phosphate + HPSA	% MeOH
0	100	0
3	92	8
30	88	12
35	82	18
40	70	30
45	70	30
55	40	60

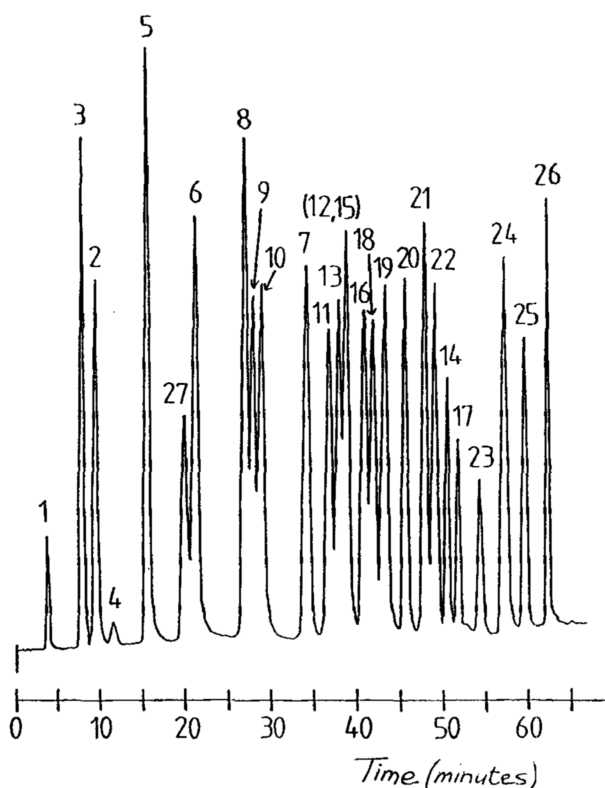

Figure 5. The chromatogram obtained using the solvent program given in Table 5. Alternative concentrations of 0.5 and 1.0 mM HPSA are used in various parts of the program. The numbering of the compounds is as in Figure 1. **27** is the second SAM hydrolysis product.

For example, the SISX and SB plots merge over much of the middle of the [HPSA] range and SISM, which is generally well removed from SISX and SB, is also coeluted. Unfortunately, several such occurences can be seen over the [HPSA] range examined and runs at intermediate [HPSA]s were generally disappointing.

However, at low [HPSA] few coelutions remained. In 1 mM HPSA there were only two and the crowded central section of the chromatogram contained many largely resolved peaks. Thus, the net result approached the best previously achieved. Hence, further low [HPSA] conditions were investigated. Table 4 gives a previously utilised binary (MEOH) gradient into which HPSA

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Table 5

Gradient Used to Generate the Chromatogram Shown in Figure 5

Time (minutes)	% 0.001 M Phosphate + 1.0 mM HPSA	% MeOH	% 0.001 M Phosphate + 0.5 mM HPSA
0	100	0	0
0.01	95	5	0
8	93	7	0
8.01	0	7	93
20	0	11	89
20.01	89	11	0
30	84	16	0
40	70	30	0
45	65	35	0
50	30	70	0

was introduced. Figures 4(a)-(d) show the chromatograms: (a) and (b) employed 0.5 mM HPSA in the aqueous phase and had exactly measured pHs of 2.86 and 2.97, respectively, whilst (c) and (d) employed 1.0 mM HPSA in the aqueous phase and had exactly measured pHs of 2.85 and 2.98, respectively. Due to the high mobility of SISM(7) in the chromatograms - both with respect to [HPSA] and pH - it is clear that the group of compounds 7-15 are better eluted in 0.5 mM HPSA. On the other hand, the SAN(3) & SG(2) pair and the group of compounds 14, 21, 22 & 17 are far better separated in 1.0 mM HPSA, and, ST(8) & SP(9) are also better resolved, albiet at some loss of resolution between SP(9) & SMRZ(10). This loss is minimised at the slightly higher pH. The separation of the second SAM hydrolysis product(27) from SDZ(6) was also favoured by the slightly higher pH.

Consequently, further chromatograms were run at the slightly higher pH, using gradients modified to incorporate the benefits of both the 0.5 and 1.0 mM HPSA concentrations. The results were as expected, and Table 5 gives the final gradient in which the MEOH concentrations are increased to bring the total run times back from about 80 minutes to under 65. The chromatogram is given in Figure 5.

Unfortunately, one coelution remains in the crowded central section. Otherwise, the least resolved pairs are (27 & 6) and (8 & 9), $R_s \approx 1.0$; (9 & 10), (13 & 12/15) and (16 & 18), $R_s \approx 0.9$.[5] Overall, it is clearly the best SFA separation obtained to date.

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