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SEPARATION OF N-ALKYLANILINES BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of the homologous N-alkylanilines on Hypersil ODS has been examined using two mobile phases, methanol–phosphate buffer, pH 8.5 (40:60, v/v) and methanol–water–1% phosphoric acid–*n*-hexylamine (30:70:100:1.4, v/v/v/v) (pH 2.5). The effects of small changes in the eluent composition, pH and temperature on the capacity factors and retention indices, based on the alkylarylketone scale, were determined. The separations were also studied on a number of other ODS-silica column packing materials. It appeared that in the methanol–phosphate buffer system there was negligible silanophilic interaction of the anilines with the column.

INTRODUCTION

Many biologically active compounds of particular interest in the life sciences are amines. Unfortunately, the analysis of such compounds by reversed-phase high-performance liquid chromatography (HPLC) has often been reported to give poor peak shapes or irreproducible peak retentions. Many of these problems are believed to arise from the silanophilic interactions of the basic amines with the acidic silanol groups left uncapped on the silica surface following the bonding of the alkyl phase[1]. Previous authors have shown that the retentions of basic compounds can be very susceptible to the presence of small amounts of aliphatic amines in the mobile phase[2–4] or to ion-pair reagents such as alkyl sulphonic acids, and both methods are often used to obtain reproducible separations.

It has been suggested that the specific interactions of basic compounds could be useful in the evaluation of column performance. As part of an earlier study to

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examine the interaction properties of reversed-phase packing materials with different eluents at pH 7.0, the retention indices, based on the alkylarylketone index scale[5], of a series of column test compounds (including N-methylaniline) were determined[6]. However, the results suggested that under these conditions, no specific interactions between the amine and the column were present.

This study extends the previous work with a more detailed examination of the separation of a series of N-alkylanilines as neutral and ionised species. It considers their interactions with different column materials and the effect on their retentions of small changes in the eluent conditions.

EXPERIMENTAL

Chemicals

N-Methyl-, N-ethyl-, N-*n*-propyl-, and N-*n*-butylanilines, *n*-hexylamine and the retention index standards (acetophenone, propiophenone, butyrophenone and valerophenone) were laboratory grade from a number of sources.

Sodium dihydrogen phosphate and disodium hydrogen phosphate analytical reagent grade and methanol HPLC grade were from Fisons Scientific Apparatus, Loughborough, U.K.

Equipment

HPLC separations were carried out using a Pye-Unicam PU 4010 pump and an Altex 153 fixed-wavelength detector set at 254 nm with a guard column packed with Hypersil. Samples (10 μl) were injected using a Rheodyne 7125 valve onto a 5-μm Hypersil ODS column (Batch 10-1229, Shandon Southern Products, Runcorn, U.K.) and were eluted with the mobile phase at 2 ml min^{-1}. Except for the early studies, the column was thermostated at 30°C. The column void volume was determined using a solution of sodium nitrate (6 mg ml^{-1}). Retention times were recorded using a Hewlett-Packard 3390 integrator.

Eluent A was methanol–phosphate buffer, pH 8.5 (40:60, v/v). The buffer was prepared by dissolving 1.56 g sodium dihydrogen phosphate dihydrate and 12.78 g disodium hydrogen phosphate in 1000 ml water.

Eluent B was methanol–water–1% phosphoric acid–*n*-hexylamine (30:70:100:1.4, v/v/v/v), pH 2.5.

Methods

The N-alkylanilines (N-methyl- to N-*n*-butyl-) and the alkylarylketones (acetophenone to valerophenone) were analysed in triplicate using both eluents. The retention indices (RI) of the alkylanilines were determined as described previously[5] based on RI_{ketone} = (carbon number) × 100.

RESULTS AND DISCUSSION

The separations of the N-alkylanilines were examined using two eluent systems. Firstly, methanol–water at pH 8.5 in which the N-alkylanilines should be neutral analytes and secondly, methanol–phosphoric acid at pH 2.5 containing *n*-hexylamine. An important feature of both systems was that the pH was controlled and

thus the degrees of ionisation of the column surface and of the analytes were fixed. Previously, variable results with basic compounds have seemed to occur primarily when the ionisation of the amine was uncontrolled and there was a mixed mode of interaction with both partition and ion-exchange mechanisms. Throughout the study, the retentions were determined as both capacity factors and retention indices, relative to the alkylarylketone scale. The latter scale is largely independent of small changes in eluent composition and is thus a guide to selectivity changes which would otherwise be masked by overall retention changes.

Separations at pH 8.5

Using methanol–phosphate buffer pH 8.5 as the eluent the retentions of N-methyl-, N-ethyl- and N-*n*-propylaniline were measured five times on separate days at ambient temperature. The capacity factors and retention indices were highly reproducible demonstrating the reliability of the method (*e.g.*, N-methylaniline: mean RI = 788.2, S.D. = 1.9). In order to test the effect of small changes in operating conditions, the analyses were repeated under a range of slightly different conditions (Table I). On changing the eluent composition from 30% to 50% methanol the capacity factors of the N-alkylanilines decreased and, as expected, the logarithm of the capacity factors of the anilines showed a linear relationship with the methanol composition of the eluent over the limited range studied (correlation coefficient 0.9995–

TABLE I

EFFECT OF CHANGES IN CHROMATOGRAPHIC CONDITIONS ON THE CAPACITY FACTORS AND RETENTION INDICES OF N-ALKYLANILINES IN METHANOL–PHOSPHATE BUFFER (pH 8.5)

Standard eluent: methanol–phosphate buffer, pH 8.5 (40:60, v/v). PhNHMe = N-methylaniline; PhNHEt = N-ethylaniline; PhNHPr = N-propylaniline; PhNHBu = N-butylaniline.

Variation	*Capacity factor*				*Retention index*			
	PhNHMe	*PhNHEt*	*PhNHPr*	*PhNHBu*	*PhNHMe*	*PhNHEt*	*PhNHPr*	*PhNHBu*
Methanol (%), ambient temperature								
30	7.31	15.99	46.32		774	852	959	
35	5.32	11.17	30.55		780	859	967	
40	3.95	7.96	20.75		788	868	976	
45	2.81	5.42	13.08		798	880	989	
50	2.02	3.72	8.38		799	880	989	
Temperature (°C), standard eluent								
10	4.73	9.41	25.05	66.92	785	861	969	1078
20	4.16	8.34	21.72	56.20	788	866	974	1081
25	3.82	7.67	19.72	50.15	788	867	975	1082
30	3.54	7.12	18.09	45.67	790	871	980	1088
40	3.08	6.19	15.12	36.24	792	875	983	1087
Eluent pH, temperature, 30°C; standard eluent								
9.73	3.58	7.20	18.17	45.22	788	870	977	1083
8.52	3.54	7.12	18.09	44.52	790	871	980	1088
7.58	3.53	7.07	17.87	44.38	789	870	978	1084

0.9997). As found previously for other neutral analytes the retention indices of the N-alkylanilines showed only a limited change with eluent composition. Although the N-alkylanilines are homologues, the difference in retention indices between N-methyl and N-ethyl aniline is only 70 units suggesting that N-methylaniline is displaying the slightly abnormal retention behaviour often observed in the first member of a homologous series. The capacity factors of N-methyl- to N-*n*-butylaniline showed a linear relationship with the reciprocal of the absolute temperature over the range 10–40°C, but again only very small changes were found in the retention indices. Changing the pH of the eluent over the range 7.58–9.73 had only a small effect on the capacity factors and virtually no effect on the retention indices, indicating that within this range the analytes are effectively neutral. Increasing the ionic strength also had only a small effect on the retentions.

These minor changes in the retention values of the N-alkylanilines are comparable to the small differences in the retentions of the neutral column test compounds (nitrobenzene, toluene, *p*-cresol and 2-phenylethanol) which were determined as part of a study of this eluent system for the analysis of barbiturates[7]. This suggests that the N-alkylanilines are effectively behaving as neutral analytes with no specific interactions with the column material.

Effect of different columns on the elution at pH 8.5

One of the major sources of irreproducibility in reversed-phase liquid chromatography results from differences between the retention properties of different batches of the same packing material or of nominally equivalent column packing materials from different manufacturers. These differing properties are a major cause of poor interlaboratory reproducibility in HPLC and the effects are reported to be particularily apparent with basic compounds[1]. The retentions of the N-alkylanilines on four columns prepared from different batches of Hypersil ODS (manufactured over a 4-year period) were therefore compared (Table II). Both the capacity factors and retention indices were very consistent. Previously different batches of this packing material were found to have very reproducible retention properties[8] and unlike the earlier reports of the separation of anilines, which used a methanol–water eluent of uncontrolled pH, in this study the mobile phase was buffered.

The separation was then repeated using columns containing packing material

TABLE II

CAPACITY FACTORS AND RETENTION INDICES OF N-ALKYLANILINES IN METHANOL–PHOSPHATE BUFFER (pH 8.5) ON FOUR DIFFERENT BATCHES OF HYPERSIL ODS

Eluent: methanol–phosphate buffer, pH 8.5 (40:60, v/v).

Compound	*Capacity factor*			*Retention index*		
	Mean	*S.D.*	*R.S.D. (%)*	*Mean*	*S.D.*	*R.S.D. (%)*
N-Methylaniline	3.49	0.16	4.6	788.8	1.50	0.19
N-Ethylaniline	6.99	0.38	5.4	870.0	1.16	0.13
N-Propylaniline	17.60	1.10	6.3	978.0	1.41	0.14
N-Butylaniline	43.93	3.24	7.4	1085.3	2.06	0.19

from five different manufacturers, chosen to include columns of very different properties ranging from Partisil ODS, which has a particularily high proportion of uncapped silanols, to Zorbax ODS. The capacity factors of the N-alkylanilines showed a wide range on the different columns emphasising the problems that can occur if attempts are made to compare results from different laboratories which use different packing materials (Table III). In marked contrast, the retention indices of the N-alkylanilines on the different columns were very consistent (*i.e.* N-methylaniline: RI = 778, S.D. 12) (Table III) and the variances are comparable to the values found earlier for neutral column test compounds on the same set of columns[8].

These results contrast with the reported large changes in selectivity towards aniline on different column materials using unbuffered methanol–water eluents[1]. Thus despite the range of experimental conditions it appears that by using retention indices and a buffered eluent very consistent and reproducible results for these weakly basic aromatic amines can be obtained. A similar result was reported by Ryba, who found that in contrast to large changes in the retentions of strongly basic amines, the retentions of aromatic amines were unaltered by the addition of dimethylformamide as a masking agent to a buffered eluent[9].

Separations at pH 2.5 with an eluent containing n-hexylamine

One method which has been widely used to reduce the silanophilic interaction of basic compounds with the stationary phase is the addition of an aliphatic amine to the eluent which is believed to coat the surface of the stationary phase and neutralise free silanol groups[2,3]. In order to test the effect of this approach on the chromatography of the N-alkylanilines, a study was carried out using an eluent containing 0.7% (v/v) *n*-hexylamine at pH 2.5, which was under investigation for the analysis of local anesthetic drugs[10,11]. In this case the N-alkylanilines are completely protonated and thus this analysis enables the interaction of the ionised amines with the column material to be examined.

The effects of changing the proportion of methanol and *n*-hexylamine in the

TABLE III

CAPACITY FACTORS AND RETENTION INDICES OF N-ALKYLANILINES IN METHANOL–PHOSPHATE BUFFER (pH 8.5) ON ODS-SILICA FROM DIFFERENT MANUFACTURERS

Eluent: methanol–phosphate buffer, pH 8.5 (40:60, v/v). Abbreviations as in Table I.

Column material	*Capacity factor*				*Retention index*			
	PhNHMe	*PhNHEt*	*PhNHPr*	*PhNHBu*	*PhNHMe*	*PhNHEt*	*PhNHPr*	*PhNHBu*
5 μm Hypersil ODS	3.54	7.12	18.09	45.67	790	871	980	1088
3 μm Hypersil ODS	4.04	8.21	20.80	52.53	790	872	979	1085
Techsil ODS	3.12	5.83	13.53	31.67	788	867	973	1081
Spherisorb ODS	2.77	4.88	10.42	22.89	767	848	955	1067
Zorbax ODS	6.90	13.65	33.35	82.76	765	849	959	1070
Partisil ODS	1.72	2.55	4.47	8.26	782	855	959	1072
Mean retention	3.71	7.03	16.56	39.6	778	858	965	1075
S.D.	1.97	4.22	11.21	28.95	12	11	10	8
R.S.D. (%)	53	60	67	73	2	1	1	1

eluent and of the temperature and pH on the separation of the N-alkylanilines were examined (Table IV). In the absence of *n*-hexylamine, but at pH 2.5, the retention indices of the N-alkylanilines were smaller than in the earlier study at pH 8.5. This can be largely attributed to the increased polarity of the protonated amino group. However, the peak shapes were very poor with considerable tailing suggesting that some retention was also occurring by an ion-exchange interaction with the stationary phase. On the addition of *n*-hexylamine the retentions of the N-alkylanilines decreased markedly and the peak shapes became more symmetrical. Presumably the *n*-hexylamine was neutralising the ion-exchange sites on the silica surface. In contrast, the capacity factors of the alkylarylketones and neutral column test compounds were largely unchanged[10]. Above 0.35% the concentration of *n*-hexylamine had only a limited further influence on the capacity factors and retention indices. The pH of the mobile phase and the temperature also had a marked influence on both the capacity factors and the retention indices of the N-alkylanilines presumably by altering the degree of ionisation.

Because *n*-hexylamine is considered to coat the surface of the stationary phase it might therefore be expected to mask any differences in the number of free silanols between different column packing materials. Repeated assays on different batches of

TABLE IV

EFFECT OF CHANGES IN THE CHROMATOGRAPHIC CONDITIONS ON THE CAPACITY FACTORS AND RETENTION INDICES OF N-ALKYLANILINES IN METHANOL–PHOSPHATE BUFFER (pH 2.5) CONTAINING *n*-HEXYLAMINE

Abbreviations as in Table I.

Variation	*Capacity factor*				*Retention index*			
	PhNHMe	*PhNHEt*	*PhNHPr*	*PhNHBu*	*PhNHMe*	*PhNHEt*	*PhNHPr*	*PhNHBu*
Hexylamine (%, v/v)								
0	2.37	3.82	8.63	20.44	565	607	680	757
0.35	0.18	0.36	1.28	3.97	362	425	541	643
0.7*	0.22	0.34	1.27	3.90	380	418	539	641
1.40	0.13	0.21	0.83	2.57	345	390	517	622
Methanol (%)								
10	0.27	0.46	1.86	5.93	374	421	545	647
15*	0.22	0.34	1.27	3.90	380	418	539	641
20	0.19	0.27	0.94	2.73	376	410	530	633
Temperature (°C)								
10	0.19	0.34	1.14	3.61	334	386	495	599
30*	0.22	0.34	1.27	3.90	380	418	539	641
40	0.26	0.37	1.42	4.26	404	437	563	665
Eluent pH								
2.0	0.12	0.24	0.83	2.64	320	384	498	603
2.5*	0.22	0.34	1.27	3.90	380	418	539	641
3.0	0.47	0.59	2.28	6.86	450	470	593	694

* Standard eluent: methanol–water–1% phosphoric acid–*n*-hexylamine (pH 2.5) (30:70:100:1.4, v/v/v/v) at 30°C.

TABLE V

CAPACITY FACTORS AND RETENTION INDICES OF N-ALKYLANILINES IN METHANOL–PHOSPHATE BUFFER (pH 2.5) CONTAINING *n*-HEXYLAMINE ON ODS-SILICA FROM DIFFERENT MANUFACTURERS

Eluent: methanol–water–1% phosphoric acid–*n*-hexylamine, (pH 2.5) (30:70:100:1.4, v/v/v/v) at 30°C.

Column material	*Capacity factor*				*Retention index*			
	PhNHMe	*PhNHEt*	*PhNHPr*	*PhNHBu*	*PhNHMe*	*PhNHEt*	*PhNHPr*	*PhNHBu*
Hypersil ODS	0.22	0.34	1.27	3.90	380	418	539	641
Partisil ODS	0.21	0.35	0.65	1.24	356	422	501	584
Zorbax ODS	0.40	0.63	2.17	6.35	309	352	470	573

the Hypersil ODS showed few differences. However, if the analysis was carried out using Partisil ODS and Zorbax ODS columns there were marked differences in both the capacity factors and retention indices of the N-alkylanilines (Table V) whereas neutral column test compounds had virtually the same indices on each column[10].

Thus, in agreement with other workers, it appears that the silanophilic interactions of reversed-phase packing materials are not completely masked by the addition of aliphatic amines to the mobile phase[12].

CONCLUSIONS

The retention indices of the N-alkylanilines show that if amines are not ionised they behave as neutral analytes without any specific interactions with the column material. However, if the amines are protonated, ion-exchange retentions can also occur which, although largely masked by the addition of an aliphatic amine to the eluent, are still susceptible to differences in the nature of the surface of the bonded phase packing material.

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