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RETENTION PREDICTION OF ANALYTES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY BASED ON MOLECULAR STRUCTURE

II. LONG TERM REPRODUCIBILITY OF CAPACITY FACTORS AND RETENTION INDICES

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SUMMARY

As part of the compilation of a database of substituent parameters for the prediction of retention indices in reversed-phase high-performance liquid chromatography, the reproducibility of retention measurements over a two-year period has been determined. Care was taken to ensure constant mobile phase and operating conditions and all the work was carried out using a single batch of stationary phase. Retention indices, based on the alkyl aryl ketones, were found to be much more consistent than capacity factors for recording retentions.

INTRODUCTION

Many methods have been proposed for the prediction of retention in high-performance liquid chromatography (HPLC) based either on comparisons with known compounds or by extrapolation from gradient elution but most do not take into account the nature of the analyte. As described in the preceding paper[1], a method has been developed to predict the retention of a compound in reversed-phase (RP) HPLC from its molecular structure and the composition of the mobile phase. The calculation is based on the summation of the retention index of a parent structure and contributions from the substituents and any interactions between the substituents.

The initial stages of the study have involved the accumulation of a database of values for substituent and interaction contributions based on experimental retention indices of model compounds[1–3]. To be useful for retention prediction all the values which contribute to the database must form a consistent data set. The individual retentions and the selectivity of the separation must therefore remain constant over the period of the study. Thus the measurement of retention must be reproducible and robust to minor changes, such as might occur in the preparation of eluents on different days, on repacking columns or to minor differences in the design of chromatographs. Because capacity factors are very susceptible to changes in the operating

conditions, retention indices based on the alkyl aryl ketones[4] were used as the basis of the study. These have been used in earlier studies to compare the reproducibility of collaborative studies[5] and the repeatability of drug assays under controlled conditions[6].

The present paper reports a study of the variations in the retentions of a number of test compounds in a wide range of eluent compositions over a two-year period using a number of replicate columns. In particular the effects of the determination of the column void volume have been examined and the use of capacity factors and retention indices as methods for recording retentions have been compared. Few previous studies have examined the robustness of HPLC retentions over a prolonged period and frequently collections of retention values have been reported without any indication of the reliability and consistency or of the expected uncertainty margins around the results.

EXPERIMENTAL

The equipment, chemicals and methods were as described in the preceding paper[1].

DISCUSSION

In the measurement of the retention values of model compounds for the prediction system, a number of steps were taken to control the experimental separation conditions to enhance long term reproducibility. The column temperature was maintained at 30°C as the capacity factors (k') of most analytes are inversely proportional to the temperature of the column[7]. Although retention indices are more robust to changes in temperature, some variations can occur due to changes in the selectivity of the separation[6]. However, in many laborartories separations are carried out at ambient temperature even though the variations within a working day can cause major changes in retention times.

The retention of ionisable compounds is dependent on the pH and ionic strength of the mobile phase. In order to control ionisation, the aqueous phase component of the mobile phase was a phosphate buffer with pH 7, which was prepared by weight from solid components to ensure constant ionic strength and pH. Most compounds, including aromatic amines and phenols are not ionised at this pH but it was not possible to examine the retention of carboxylic or sulphonic acid groups. The retentions of neutral compounds were largely unaffected on changing the strength of the buffer in steps from 0.00 to 0.02 M in methanol–buffer (70:30) and acetonitrile–buffer (70:30) eluents so that the exact buffer concentration would not be a critical factor. However, the retentions of strongly basic primary amines decreased considerably with increases in ionic strength (3-phenylpropylamine, pK_a = 10.39, in acetonitrile–buffer (70:30); 0.001 M, k' = 24.23; 0.005 M, k' = 8.79; 0.02 M, k' = 4.44). These changes suggested that the amines were partially or fully protonated and were being retained by an ion-exchange interaction with the acidic silanols on the surface of the silica rather than a reversed-phase partition mechanism[8]. The aliphatic amino group was therefore also not included in the present study, although it is planned in the future to examine the unionised acidic and basic groups by using different pH buffers.

Large changes in capacity factors and to a lesser extent retention indices have been reported for separations on different brands of nominally equivalent column packing materials and significant differences can also occur when different batches of the same manufacturer's packing material have been used[6]. To ensure consistent results within the study, a single batch of Spherisorb ODS-2 was used throughout. So far five columns (A–E) have been used, they were repacked with fresh stationary phase as soon as the peak shapes started to deteriorate or the retentions of standard compounds altered. The capacity factors of a number of compounds were examined in the same eluent on three of the columns. These showed some moderate variations [relative standard deviations (R.S.D.) of up to 9%] but the corresponding retention indices were more consistent with a variation of less than three units for rapidly eluted compounds and approximately one unit for well retained compounds (Table I). These variations in retention indices were no greater than the differences between individual replicate separations on a single day.

TABLE I

CAPACITY FACTORS OF A SELECTION OF COMPOUNDS DETERMINED ON THREE COLUMNS USED IN THE STUDY

Eluent: methanol–buffer (60:40).

Compound	*Capacity factor*			*Mean*	*S.D.*	*Retention index*			*Mean*	*S.D.*
	Column					*Column*				
	A	*B*	*C*			*A*	*B*	*C*		
Acetophenone	1.59	1.59	1.54	1.57	0.03					
Propiophenone	2.88	2.96	2.74	2.86	0.11					
Butyrophenone	4.93	5.18	4.64	4.92	0.27					
Valerophenone	8.94	9.62	8.40	8.99	0.61					
Hexanophenone	16.61	18.23	15.57	16.80	1.34					
Heptanophenone	31.70	34.85	29.38	31.98	2.74					
Phenylacetamide	0.52	0.46	0.47	0.48	0.03	605	600	603	603	2.5
Benzyl alcohol	0.82	0.79	0.78	0.80	0.02	694	689	689	691	2.9
Benzyl cyanide	1.21	1.20	1.15	1.19	0.03	758	756	755	756	1.5
Methyl phenylacetate	2.53	2.30	2.15	2.33	0.19	861	863	862	862	1.0
Toluene	6.52	6.76	6.08	6.45	0.35	1041	1038	1039	1039	1.5
Benzyl bromide	5.74	6.01	5.44	5.73	0.29	1020	1019	1020	1020	0.6

Column void volume

Capacity factors are calculated from the equation $k' = (t_R - t_0/t_0)$. However, small variations in the measured column void volume could significantly alter the calculated capacity factors but despite its importance, there is still no agreed standard method for its determination. Numerous suggestions have been compared but no single method is generally applicable[9,10]. In contrast, we have previously noted that the value of column void volume has only a small effect on the retention indices if the analyte is eluted within the calibrated range[11].

In the present study a 10-μl injection of dilute aqueous solution of sodium nitrate (6 mg ml^{-1}) has been used to determine the column void volume. This marker

compound is readily detectable spectroscopically but it has previously been noted that a fixed concentration should be used[12,13]. This was confirmed using 10 μl injections of aqueous sodium nitrate solutions containing 1–24 mg ml^{-1} sodium nitrate, corresponding to molar concentrations of 0.01–0.28 *M* sodium nitrate. As the concentration increased, the retention times measured at the peak maximum increased from 0.843 to 0.922 min with methanol–buffer (70:30) and from 0.749 to 0.818 min with acetonitrile–buffer (70:30). If the eluent buffer strength was altered there were only minor changes.

There is reported to be a direct relationship between the retention of a series of homologues and retention such that the zero retention index should represent the column void volume[9]. Therefore as an alternative to the direct measurement of the column void volume, two calculation methods have been examined using the homologous alkyl aryl ketone standards. The first is a method proposed by Berendsen *et al.*[14] and the second an iterative procedure developed by Smith and Garside[15]. However, in both cases the results were very erratic and often the calculated column void volume was greater than the retention times of rapidly eluting analytes.

In order to determine how much variation could be expected in the final retention results as a consequence of differences in the measurement of the column void volume, the individual experimental values at each eluent composition during the two years of the study have been evaluated. In each case there were between 20 and 30 measurements and they showed variations of up to 20% from the mean value [*i.e.*, methanol–buffer (50:50), t_0 ranged from 0.568 to 0.678 min with an R.S.D. of 15%]. To determine the effects of variations of this magnitude the capacity factors and retention indices for a number of compounds using high (90%) and low (40%) percentage of modifier were recalculated from the experimental retention times by assuming the mean column void volume and values which were 15% higher and lower. The compounds covered the range of alkyl aryl ketones and included compounds eluting before and within the calibrated region of the retention index scale (Table II). As expected, the calculated capacity factors were very dependent on the value of the column void volume, particularly when the retention times were similar to the column void volume. This confirmed the sensitivity of capacity factors to the exact value of the column void volume and their susceptibility to different methods of measurement and consequently low reliability for comparison studies.

The retention indices (*RI*) in 40% modifier were generally robust and even for the rapidly eluted compound, phenylacetamide, varied by only seven units. With 90% organic modifier the retention indices for all the rapidly eluted compounds with retentions shorter than acetophenone and therefore outside the calibration region, showed marked variations (phenylacetamide from $RI = 605$ to 455 and 3-phenyl-1-propanol from 755 to 719). However, for all these compounds the capacity factors were less than 0.5. Thus at these very low retentions any minor variations in the measured column void volume could have a marked effect on the reproducibility of retention indices. Similar results were obtained for the corresponding eluents containing acetonitrile as the modifier.

These calculations represent the worst possible examples and indicate the maximum extent of expected variations. Clearly if the capacity factors of analytes are small (particularly if the retention indices are less than 700) there is likely to be a considerable uncertainty in the values. Thus the use of retention indices while largely

TABLE II

EFFECT OF CHANGES IN THE VALUE OF THE COLUMN VOID VOLUME USED TO CALCULATE CAPACITY FACTORS AND RETENTION INDICES

Compound	*Capacity factor*			*Retention index*		
	Assumed column void volume			*Assumed column void volume*		
	−15%	*Mean*	*+15%*	*−15%*	*Mean*	*+15%*
Column void volume (min)[a]	0.341	0.401	0.461	0.341	0.401	0.461
Acetophenone	9.29	7.75	6.61	804	804	803
Hexanophenone	270.04	229.49	199.49	1197	1197	1197
Phenylacetamide	2.37	1.87	1.49	645	638	631
Benzyl alcohol	3.74	3.03	2.50	698	694	691
2-Phenylethanol	6.31	5.22	4.41	759	758	756
3-Phenyl-1-propanol	12.67	10.62	9.11	840	840	840
4-Phenylbutyronitrile	22.22	18.74	16.17	906	906	906
Toluene	93.85	79.66	69.16	1074	1074	1075
Column void volume (min)[b]	1.404	1.652	1.900	1.404	1.652	1.900
Acetophenone	0.75	0.48	0.29	810	804	795
Hexanophenone	1.52	1.15	0.87	1196	1197	1199
Phenylacetamide	0.51	0.28	0.12	605	560	455
Benzyl alcohol	0.57	0.33	0.16	665	635	574
2-Phenylethanol	0.61	0.37	0.19	705	683	643
3-Phenyl-1-propanol	0.67	0.42	0.24	755	741	719
Methyl 3-phenylpropionate	0.94	0.65	0.43	809	803	794
Toluene	1.31	0.96	0.70	1113	1117	1122

[a] Eluent: Methanol–pH 7 buffer (40:60) (2 ml min^{-1}).
[b] Eluent: Methanol–pH 7 buffer (90:10) (0.5 ml min^{-1}).

compensating for variations in capacity factors does not totally overcome the problem of the reproducibility of the measurements of the column void volume. On elution with 90% modifier many analytes had particularly low capacity factors (often $k' < 0.5$) and these often required extrapolation beyond the range of retention index standards. In addition, there was doubt that the retentions at the high organic proportions could be directly related to lower compositions[1]. Consequently, the range of the study has been limited to eluents up to 80% modifier. In addition, in subsequent work the retentions of compounds with capacity factors lower than 0.15 in any eluent have also been excluded from the calculations.

Long term reproducibility

Despite controlling as many factors as feasible, experimental variation cannot be totally eliminated. This will be reflected in the uncertainty in any determined value and therefore in the accuracy which could be expected from the predicted retention indices. To examine the success of the precautions taken to produce reproducible retentions and to determine the anticipated uncertainty in an individual retention index value, an investigation of the long term reproducibility was undertaken.

Apart from the earliest part of the work on the first column, the retention times

TABLE III

LONG TERM REPRODUCIBILITY OF CAPACITY FACTORS AND RETENTION INDICES OF TOLUENE, BENZENE AND PHENOL OVER A TWO-YEAR PERIOD

Methanol–buffer	*Capacity factor*				*Retention index*			
	Mean	*Max.*	*Min.*	*S.D.*	*Mean*	*Max.*	*Min.*	*S.D.*
Phenol								
40:60	2.24	2.80	2.02	0.21	691	704	685	5.4
50:50	1.26	1.29	1.14	0.10	689	695	681	7.0
60:40	0.71	0.83	0.75	0.02	683	689	679	2.7
70:30	0.53	0.57	0.50	0.02	673	679	669	3.2
80:20	0.34	0.39	0.33	0.02	654	663	648	5.4
Benzene								
40:60	12.27	13.22	11.38	0.60	885	891	883	5.6
50:50	6.56	6.99	5.91	0.50	913	917	909	4.0
60:40	3.55	3.86	3.22	0.15	936	941	933	2.0
70:30	1.97	2.09	1.75	0.07	956	962	952	2.0
80:20	1.11	1.21	1.06	0.04	980	986	977	2.8
Toluene								
40:60	29.83	31.80	27.25	1.07	986	989	979	5.7
50:50	13.92	16.07	12.32	0.90	1015	1022	1012	3.7
60:40	7.13	7.81	5.54	0.84	1036	1045	1031	4.3
70:30	3.24	3.46	2.40	0.28	1063	1065	1059	1.8
80:20	1.66	1.80	1.56	0.07	1091	1097	1088	2.9
Acetonitrile–buffer								
Phenol								
30:70	2.46	2.84	1.84	0.21	695	697	688	2.2
40:60	1.51	1.64	1.38	0.07	688	696	682	3.2
50:50	0.96	1.09	0.84	0.18	672	682	664	4.4
60:40	0.65	0.68	0.63	0.02	660	661	658	1.2
70:30	0.44	0.47	0.42	0.01	641	642	638	2.1
80:20	0.31	0.39	0.24	0.05	625	652	611	11.6
Benzene								
30:70	13.76	15.38	11.99	1.02	905	910	899	3.9
40:60	6.90	7.79	6.14	0.57	926	932	923	4.4
50:50	3.70	4.27	2.98	0.37	939	941	931	2.9
60:40	2.14	2.28	2.03	0.10	949	952	944	2.2
70:30	1.31	1.40	1.29	0.05	956	959	951	3.5
80:20	0.84	0.95	0.74	0.06	962	969	958	3.8
Toluene								
30:70	29.75	31.98	25.27	2.19	1003	1008	999	2.9
40:60	12.78	14.37	11.31	1.20	1019	1026	1017	3.8
50:50	6.12	7.01	4.94	0.59	1031	1037	1024	4.4
60:40	3.18	3.41	3.01	0.15	1043	1047	1039	2.6
70:30	1.87	1.98	1.73	0.08	1051	1057	1049	3.5
80:20	1.13	1.26	1.09	0.09	1061	1065	1055	3.2

of three standard compounds, phenol, benzene and toluene were measured as part of every set of separations. This resulted in 20–30 individual measurements at each eluent composition over the two-year period depending on the number of times each eluent was used. These standard compounds were chosen to represent both polar and non-polar analytes and the two homologues could be used to check the consistency of the methylene selectivity. The range, mean and standard deviations from the mean of the capacity factors were determined for the standards (Table III). Although the capacity factors on each column were reasonably consistent, there were significant differences between the columns even though they were all packed with the same batch of Spherisorb ODS 2 (Fig. 1). Despite the precautions taken to ensure that the experimental conditions remained as constant as possible, there is still considerable variation in the results of up to 18% from the mean values. Although much of this variation is due to the different columns, there are also variations resulting from uncertainties in measuring both the retention times and the column void volume.

Generally the retention indices of the three standards were more consistent and suggest that retention indices can be expressed with a high degree of confidence as the measured value ± 10 units (twice the standard deviation) (Table III). No significant differences could be seen in the results from the different columns (Fig. 2).

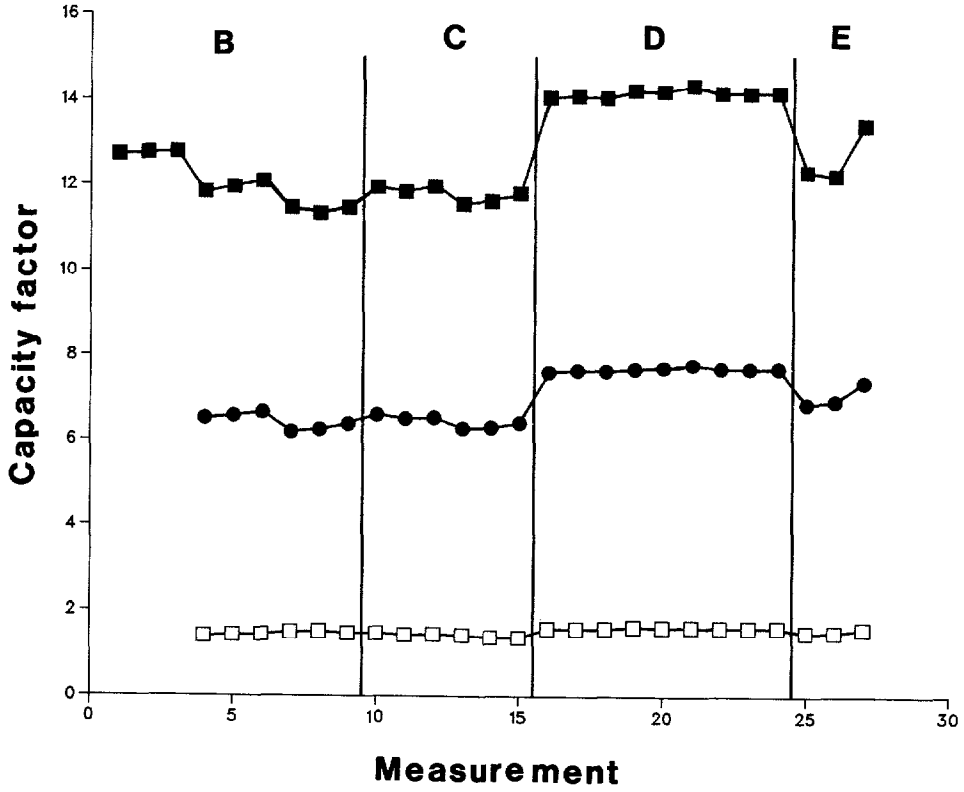

Fig. 1. Individual measurements of capacity factors of phenol (□), benzene (●) and toluene (■) throughout the study using acetonitrile–buffer (40:60) as the mobile phase on four different columns (B–E) packed with the same batch of Spherisorb ODS-2.

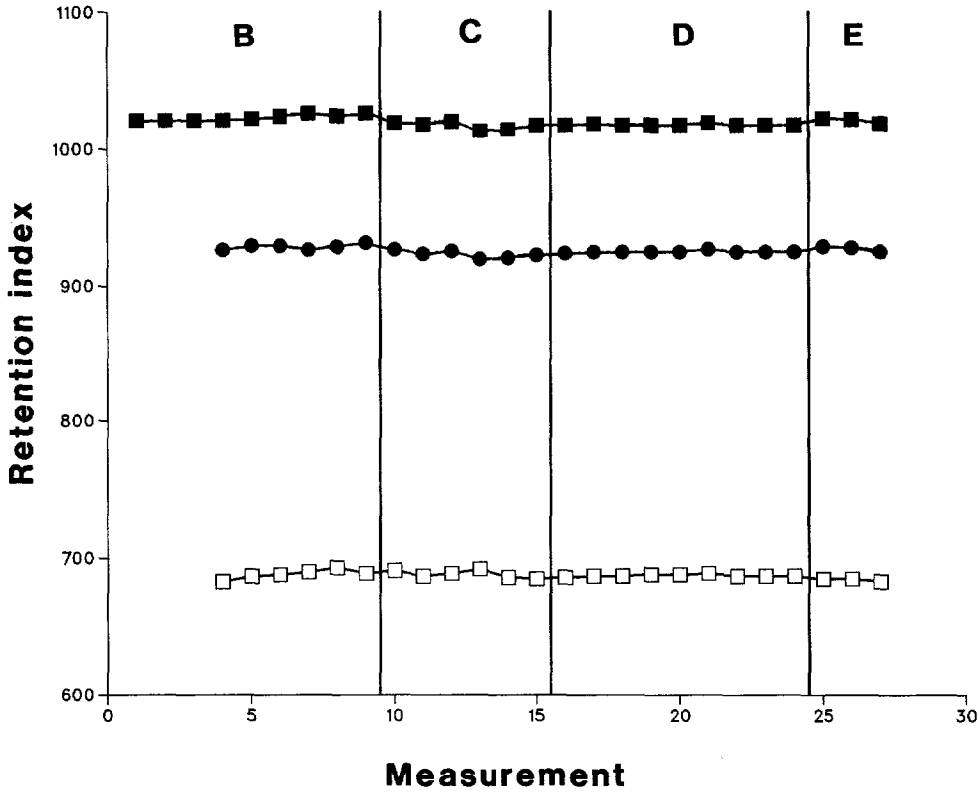

Fig. 2. Individual measurements of retention indices of phenol (□), benzene (●) and toluene (■) throughout the study using acetonitrile–buffer (40:60) as the mobile phase on four different columns (B–E) packed with the same batch of Spherisorb ODS-2.

CONCLUSION

The variation in the capacity factors over a prolonged period even under closely controlled chromatographic conditions is considerable and is emphasised by variations in the measurement of the column void volume. However, the use of relative measurements expressed as retention indices can eliminate much of the variation and can act as an efficient method for standardisation. The retention indices of many compounds can probably be expressed with high confidence to within 10 retention index units. This gives a guide to the expected precision of predicted retentions based on this data depending on the number of contributions that are included.

These conclusions have considerable relevance for other large collections of chromatographic data as even using the same batch of packing material and controlled experimental conditions, capacity factors are likely to have varied considerably unless some form of correction has been applied throughout the period of the study.

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