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RETENTION REPRODUCIBILITY OF THIAZIDE DIURETICS AND RELATED DRUGS IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ROGER M. SMITH*, GRACE A. MURILLA* and TONY G. HURDLEY
Department of Chemistry, Loughborough University of Technology, Loughborough, Leicestershire, LE11 3TU (U.K.)
and
RICHARD GILL and ANTHONY C. MOFFAT**
Central Research Establishment, Home Office Forensic Science Service, Aldermaston, Reading, Berkshire RG7 4PN (U.K.)

SUMMARY

A method has been developed for the separation of thiazide diuretics and a number of related drugs by high-performance liquid chromatography on an ODS-Hypersil column with acetonitrile–1% aqueous acetic acid as the eluent. The effects caused by changes in the separation conditions on the reproducibility and robustness of alternative methods for recording retentions (including capacity factors, retention indices based on the alkyl aryl ketone scale, and relative capacity factors compared to a thiazide standard) have been examined. The results confirm that good interlaboratory reproducibility will only be achieved when operators control the temperature of the column and use the same brand of column packing material. The retentions should be recorded using a relative method, as these were found to be virtually independent of minor variations in the eluent composition.

INTRODUCTION

The retention time of a compound in high-performance liquid chromatography (HPLC), during a single analytical determination, is usually very consistent, and a close correspondence is obtained between analytes and standards. However, retention properties are susceptible to even small changes in the chromatographic conditions, such as the proportion of the organic component, the pH or ionic strength of the eluent, or the temperature of the column. They are also very sensitive to the brand of column packing material used, even if such materials are nominally equivalent, and retentions can even differ with different batches of the same brand. As a conse-

* Present address: Government Chemist Department, P.O. Box 20753, Nairobi, Kenya.
** Present address: Home Office Forensic Science Laboratory, Hinchingbrooke Park, Huntingdon, Cambridge PE18 8NP, U.K.

quence, it is difficult to identify samples by comparisons with results from other laboratories or to build up compilations of retention values in libraries or databases. Each laboratory must, instead, calibrate each analytical system using a full set of standard compounds, or use interlaboratory databases for preliminary comparisons, followed by a direct calibration with a limited number of credible compounds for each unknown.

As part of a series of studies aimed at improving the reproducibility of retention values in HPLC so that values obtained in one laboratory can be used in another, a number of drug separations of forensic interest have been examined. This work has included detailed studies on the influence of the eluent composition, operating conditions, and the nature of the stationary phase on the retentions of barbiturates[1,2] and local anaesthetic drugs[3]. The purpose of this approach was to identify the factors that need to be closely controlled in order to obtain consistent results and to compare different methods of recording retentions[1–4]. Experience with the various drug separations showed that, for reproducible results, the pH of the eluent and the temperature of the column need to be closely controlled and, if possible, a single batch of the stationary phase (and certainly the same brand of packing material) should be used.

The most robust results were obtained when the retentions were recorded as relative values, either as retention indices compared to the alkyl aryl ketones or as relative retention times or relative capacity factors compared to a related standard. Conventionally, capacity factors [$k' = (t_R - t_0)/t_0$] have been used to record retentions in HPLC but their calculation is very dependent on the value measurement for the column void volume (t_0). As yet there is no standard method for this measurement and, although numerous different techniques have been proposed, these often give different values on the same column[5]. In practice, individual laboratories may use different techniques, while in many reports the method used is not stated. However, even small absolute changes in this value can cause large changes in the calculated values of k' from the same measured retention times.

Although Kováts retention indices, based on the *n*-alkanes have been widely used in gas–liquid chromatography (GLC) to define retentions, so far, similar concepts have not gained wide acceptance in HPLC. The first proposals were made by Baker and Ma[6], who suggested that the alkan-2-ones could be used as the basis of a scale. However, these compounds have only a weak ultraviolet absorption, and Smith[7] suggested that the homologous alkyl aryl ketones would be more readily detected. In both cases, the standards showed a linear relationship between the number of carbon atoms and log k', and the retention indices of neutral sample compounds were virtually independent of the proportion of methanol–water in eluents over a wide range. The application of retention indices based on the alkyl aryl ketone scale has subsequently been extended by the selection of a set of column test compounds the indices of which can be used to characterise column differences and mobile-phase selectivities in a similar manner to the use of McReynolds constants in GLC[8–10]. The value of relative retention measurements was confirmed by an interlaboratory collaborative study of the separation of the barbiturates[11].

As all these drug separations used aqueous methanol as eluents, studies have recently been undertaken to confirm that the retention index concept is also generally valid with other modifiers of the mobile phase, such as acetonitrile or tetrahydro-

furan[12]. This work confirmed that there was a linear relationship between the log k' of the ketones and their carbon number. The variations of the retention indices of test compounds with different proportions of acetonitrile or tetrahydrofuran suggested that these systems might be less robust and show greater changes in retention indices with the composition of the mobile phase than with methanol.

Based on this work, the present paper examines in detail the application of a separation system in which a mobile phase containing acetonitrile is used for the separation of the thiazide diuretic drugs. Previously, a number of HPLC methods have been reported for the determination of individual thiazide diuretics because of their importance as widely used antihypertensive drugs. However, most of these methods have concentrated on the quantitative determination of a single or a small group of thiazides as known constituents in a biological fluid (*e.g.* hydrochlorothiazide)[13]. A few studies have examined the identification of the thiazides[14–16], and a more detailed study has recently been reported by De Croo *et al.*[17], who examined the effect of eluent composition, organic modifier, pH, and temperature on the capacity factors of a number of diuretic drugs. In a second paper, they examined the differences between C_2, C_8 and C_{18} alkyl-bonded stationary phases for this separation[18].

The present paper has adapted a mobile phase of acetonitrile–1% aqueous acetic acid, proposed by Tisdall *et al.*[15]. Our work included a detailed examination of the reproducibility of the method and of the robustness of different approaches to recording retentions.

EXPERIMENTAL

Chemicals and eluents

Reference samples of alkyl aryl ketones (acetophenone, propiophenone, butyrophenone, and valerophenone) and column test compounds (toluene, nitrobenzene, 2-phenylethanol, N-methylaniline and *p*-cresol) were of laboratory reagent grade from a range of different suppliers. Acetonitrile was of HPLC grade (Fisons Scientific Apparatus, Loughborough, U.K.) and acetic acid was AnalaR grade (BDH Chemicals, Poole, U.K.).

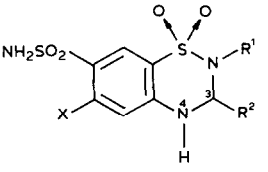
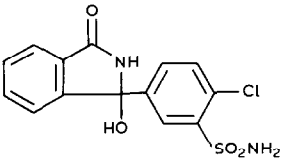
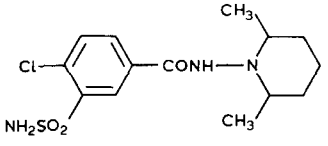
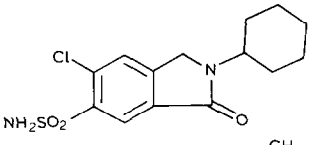
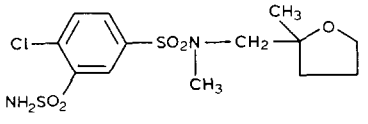
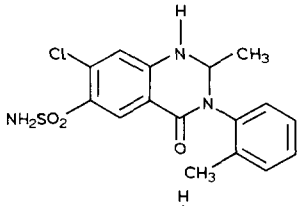
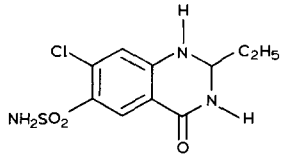
The thiazide diuretic drugs and related compounds (Table I) were from the reference collection at the Central Research Establishment, Home Office Forensic Science Service. They were made up as solutions in acetonitrile (50–100 mg/l).The following HPLC column packing materials were used: ODS-Hypersil, 5 μm, batches 10/1229 and 6/868 (Shandon Southern, Runcorn, U.K.), ODS-Zorbax (Du Pont, Wilmington, DE, U.S.A.), ODS-Techsil 5 C18, 5 μm, and ODS-Techsphere, 5 μm, (HPLC Technology, Macclesfield, U.K.), Lichrosorb RP-18 (Merck, Darmstadt, F.R.G.), Nucleosil 5 C_{18}, 5 μm, (Machery-Nagel, Duren, F.R.G.). Columns (100 × 5 mm, Shandon Southern) were prepared by slurry packing.

Routine HPLC method[19]

The separations were carried out using a M6000 pump (Waters Chromatography Division, Milford, MA, U.S.A.) and a CE272 Spectrophotometric detector (Cecil Instruments, Cambridge, U.K.), operated at 271 nm. The samples (10 μl), dissolved in acetonitrile, were injected using a 7125 valve (Rheodyne, Cotati, CA,

TABLE I

THIAZIDE DIURETICS AND RELATED DRUGS USED IN THE STUDY

Compound number	*Compound*	*Structure*	*X*	*R*[1]	*R*[2]
Thiazide diuretics					
1	Bendrofluazide		CF_3	H	$CH_2C_6H_5$
2	Benzthiazide		Cl	H	$CH_2SCH_2C_6H_5$
3	Chlorothiazide		Cl	H	(3,4-dehydro) H(3,4-dehydro)
4	Cyclopenthiazide		Cl	H	$CH_2C_5H_9$
5	Cyclothiazide		Cl	H	Norborn-5-en-2-yl
6	Hydrochlorothiazide		Cl	H	H
7	Hydroflumethiazide		CF_3	H	H
8	Methyclothiazide		Cl	CH_3	CH_2Cl
9	Polythiazide		Cl	CH_3	$CH_2SCH_2CF_3$
10	Trichlormethiazide		Cl	H	$CHCl_2$
Non-thiazide diuretic drugs					
11	Chlorthalidone				
12	Clopamide				
13	Clorexolone				
14	Mefruside				
15	Metolazone				
16	Quinethazone				

U.S.A.), fitted with a 20-μl loop, into an ODS-Hypersil column (batch 10/1229) (160 × 5 mm I.D.) at ambient temperature. The column was eluted with acetonitrile–1% aqueous acetic acid (30:70, v/v) at 2 ml/min.

Reproducibility studies

Reproducibility studies of the HPLC separation were carried out using a 4010 pump (Pye Unicam, Cambridge, U.K.) and 153 fixed-wavelength detector (Altex Scientific, Beckman Instruments, San Ramon, CA, U.S.A.), set at 254 nm. The samples (10 μl) were injected using a Rheodyne 7125 valve into an ODS-Hypersil column (batch 10/1229) (100 × 5 mm I.D.), encased in a water jacket at 30°C. The column was eluted with acetonitrile–1% aqueous acetic acid (35:65, v/v) at 2 ml/min, which had been passed through a precolumn, containing silica, placed between the pump and injection valve. The column void volume was determined by using an aqueous solution of sodium nitrate (12.16 mg/ml). The chromatographic peaks were recorded using a C-R3A integrator (Shimadzu, Tokyo, Japan).

Calculations

The retention times were determined in triplicate, and the mean values were reported. Retention indices were determined as described previously using a least-squares correlation between the logarithm of the capacity factors for the alkyl aryl ketones and their carbon numbers[7]. The retention indices are based on *RI*(ketones) = number of carbon atoms × 100. Relative capacity factors were calculated relative to polythiazide.

RESULTS AND DISCUSSION

As part of a process of rationalisation of HPLC methods within U.K. Forensic Science Laboratories, ODS-Hypersil has been selected as the standard reversed-phase column packing material to facilitate the transfer of methods and data between laboratories. A common batch of this packing material is supplied to each laboratory. The present study therefore commenced with the conversion of the method proposed by Tisdall *et al.*[15], for a μBondapak C_{18} column, to an ODS-Hypersil column.

Development of the eluent system

In their work, Tisdall *et al.*[15] showed that all the commonly used diuretics could be separated on a μBondapak C_{18} column using acetonitrile–1% aqueous acetic acid (either 8:92 or 35:65) eluents. The former, weaker eluent was primarily required to separate chlorothiazide and hydrochlorothiazide from interfering components in biological fluids and was not studied in the present work.

When the second eluent, acetonitrile–1% aqueous acetic acid (35:65) was used to separate a series of thiazide diuretics and related compounds (Table I) on the ODS-Hypersil column, the order of elution was very similar to that reported[15], except that in this case polythiazide was eluted just before bendrofluazide whereas the reverse order had been obtained on the μBondapak C_{18} column. Most of the analytes gave good peak shapes. Multiple peaks were obtained from cyclothiazide, but only the two major peaks have been noted in this report. Similar multiple peaks were also found by Tisdall *et al.*[15] and De Croo *et al.*[17] and were ascribed to the presence of

isomers. Metolazone, quinethazone, and trichlormethiazide slowly decomposed on standing in the mobile phase, and after a few days, additional peaks were observed. Therefore, fresh samples had to be prepared at frequent intervals.

It was found that isocratic eluents with acetonitrile contents in the range 30–40% gave satisfactory results for typical thiazides. From these results, acetonitrile–1% aqueous acetic acid (30:70, v/v) was selected for routine use[19], as this gave sufficient retention of the early-eluted components without unduly prolonging the assay. Typical values of the capacity factors for the thiazide diuretics and related compounds were determined at ambient temperature (Table II)[19]. Fig. 1 shows the separation of eight compounds on a 16-cm column, demonstrating the good peak shapes.

Retention reproducibility

As part of the series of studies[1–3] aimed at assessing the factors that influence reproducibility of drug separations of forensic interest, a more detailed examination was then carried out under carefully controlled conditions to investigate the retention properties of the diuretic drugs. In order to identify any factors that need to be closely controlled in the separation of the diuretic drugs, it was necessary to determine the magnitude of any effects arising from small changes in the operating conditions or eluent composition. An eluent of acetonitrile–1% aqueous acetic acid (35:65)[15], with a 10-cm column, maintained at 30°C, was selected for this work and each of the variables was altered in turn.

TABLE II

CAPACITY FACTORS AND RELATIVE CAPACITY FACTORS OF THIAZIDE DIURETICS AND RELATED DRUGS

ODS Hypersil column (160 × 5 mm); eluent, acetonitrile–1% aqueous acetic acid (30:70); ambient temperature.

Compound	*Capacity factor*	*Relative capacity factor × 100*
Thiazide diuretics		
Chlorothiazide	0.54	3.5
Hydrochlorothiazide	0.70	4.6
Hydroflumethiazide	1.30	8.6
Trichlormethiazide	3.10	20.5
Methyclothiazide	3.82	25.3
Benzthiazide	9.32	61.8
Cyclothiazide 1	10.78	71.4
Cyclothiazide 2	11.91	78.9
Polythiazide	15.09	100.0
Bendrofluazide	15.35	101.7
Cyclopenthiazide	16.45	109.0
Non-thiazide diuretic drugs		
Quinethazone	0.67	4.4
Chlorthalidone	1.28	8.5
Metolazone	4.89	32.4
Clorexolone	7.26	48.1
Mefruside	8.67	57.4
Clopamide	4.01	26.6

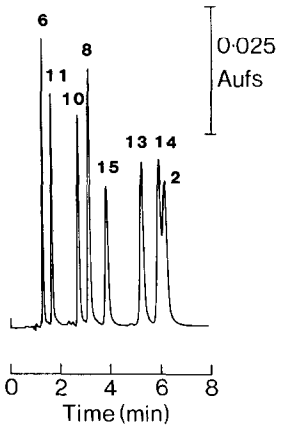


Fig. 1. Separation of diuretic drugs on ODS-Hypersil (160 × 5 mm). Eluent, acetonitrile–1% aqueous acetic acid (30:60) at 2 ml/min; ambient temperature; detection at 271 nm. Peaks correspond to Table I.

The study also set out to compare whether alternative methods to capacity factors for recording retention values might be more robust and less susceptible to the variations in conditions. One of the problems with the use of capacity factors for identifications is their susceptibility to small differences in the eluent composition and the value of the column void volume. In their paper, De Croo *et al.*[17] examined a number of alternative systems for the determination of t_0 for this assay and selected the use of ^2H_2O–acetonitrile as a marker but this requires a refractive-index detector for detection. They found that different concentrations of sodium nitrate gave dif-

TABLE III

CAPACITY FACTORS OF ALKYL ARYL KETONES AND COLUMN TEST COMPOUNDS

ODS Hypersil column (100 × 5 mm); temperature, 30°C; eluent acetonitrile–1% aqueous acetic acid (35:65).

Compound	*Capacity factor*	*Retention index*	*Retention index, acetonitrile–buffer, pH 7 (30:70)**
Retention index standards			
Acetophenone	2.76	800	800
Propiophenone	6.00	900	900
Butyrophenone	11.89	1000	1000
Valerophenone	24.13	1100	1100
Column test compounds			
N-Methylaniline	0.75	615	828
2-Phenylethanol	1.45	706	713
p-Cresol	2.30	771	776
Nitrobenzene	4.68	870	869
Methylbenzoate	5.51	888	886
Toluene	12.72	1009	996

* Comparison with retention indices obtained in an earlier study for a similar eluent[12].

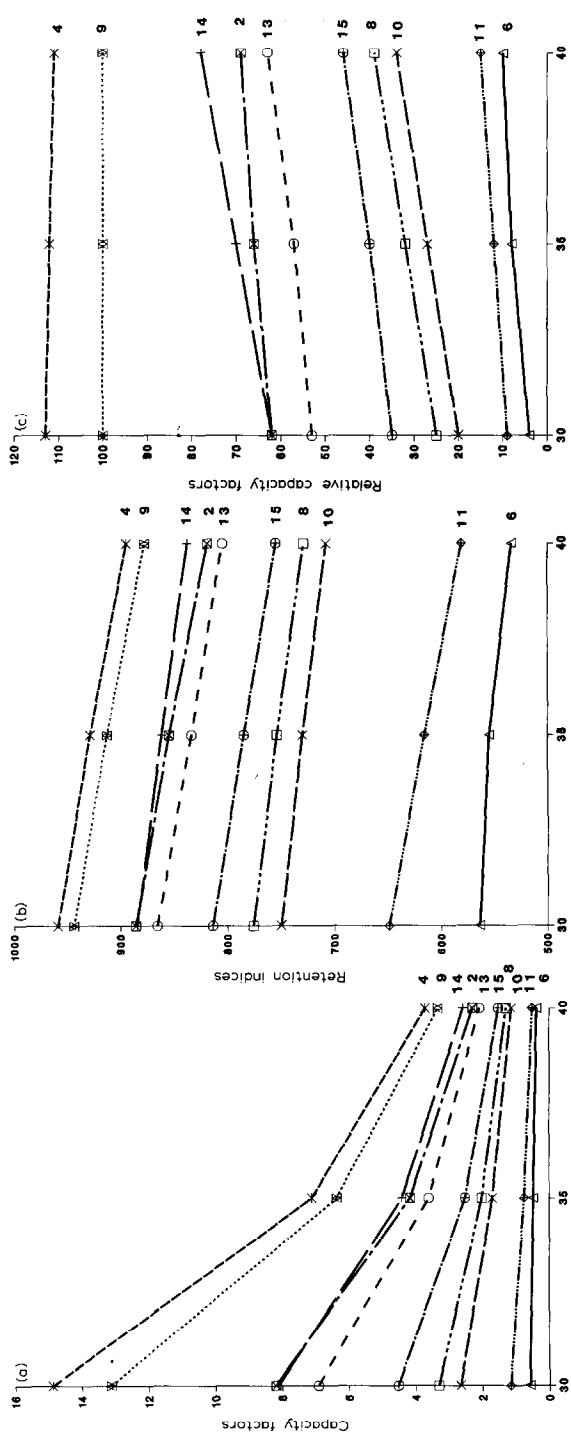


Fig. 2. Effect of the proportion of acetonitrile on (a) capacity factors, (b) retention indices and (c) relative capacity factors of selected thiazide diuretics and related drugs. Numbers correspond to Table I.

ferent values, but if a dilute solution of fixed concentration is used (as in the present study) the void volume values will be reproducible.

Separation selectivity

The alkyl aryl ketones (acetophenone–valerophenone) and the column test compounds (toluene, nitrobenzene, *p*-cresol, 2-phenylethanol, and N-methylaniline) were chromatographed with acetonitrile–1% aqueous acetic acid (35:65), and the capacity factors and retention indices were calculated (Table III). With the exception of N-methylaniline, which would be protonated in acetic acid, the indices of the column test compounds were very similar to those measured previously on the same column when acetonitrile–phosphate buffer, pH 7 (30:70) is used as the eluent[12]. The changes in pH and buffer components, therefore, appear to have little effect on the overall selectivity of the separation system. The retention indices for the column test compounds were also determined in all of the present studies but are usually not

TABLE IV

EFFECT OF THE PROPORTION OF ACETONITRILE IN THE MOBILE PHASE ON THE CAPACITY FACTORS, RETENTION INDICES, AND RELATIVE CAPACITY FACTORS OF THIAZIDE DIURETICS AND RELATED DRUGS

ODS Hpersil column (100 × 5 mm); temperature, 30°C. Relative capacity factors calculated relative to polythiazide.

Compound	*Capacity factor*			*Retention index*			*Relative capacity factor × 100*		
	Acetonitrile (%)			*Acetonitrile (%)*			*Acetonitrile (%)*		
	30	*35*	*40*	*30*	*35*	*40*	*30*	*35*	*40*
Thiazide diuretics									
Chlorothiazide	0.47	0.40	0.32	539	527	508	3.6	6.3	9.6
Hydrochlorothiazide	0.58	0.49	0.38	564	555	534	4.4	7.3	11.3
Hydroflumethiazide	1.13	0.85	0.63	645	632	615	8.6	13.3	18.8
Trichlormethiazide	2.66	1.72	1.14	750	730	708	20.3	26.9	34.0
Methyclothiazide	3.31	2.04	1.30	776	754	729	25.2	31.9	38.8
Benzthiazide	8.19	4.19	2.31	886	855	819	62.4	65.6	69.0
Cyclothiazide 1	9.38	4.78	2.85	903	873	852	71.4	74.8	85.1
Cyclothiazide 2	10.28	5.21	3.52	914	885	886	78.3	81.5	105.1
Polythiazide	13.13	6.39	3.35	944	913	878	100.0	100.0	100.0
Bendrofluazide	13.53	6.60	3.45	948	918	883	103.0	103.3	103.0
Cyclopenthiazide	14.87	7.13	3.73	959	929	895	113.3	111.6	111.3
Non-thiazide diuretic drugs									
Quinethazone	0.61	0.46	0.35	570	546	522	4.6	7.2	10.4
Chlorthalidone	1.16	0.76	0.51	649	616	581	8.8	11.9	15.2
Metolazone	4.52	2.54	1.53	814	785	755	34.4	39.8	45.7
Clorexolone	6.92	3.63	2.10	866	834	805	52.7	56.8	62.7
Mefruside	8.11	4.43	2.60	885	862	838	61.8	69.3	77.6
Clopamide	4.34	3.64	2.64	809	835	841	33.1	57.0	78.8
Column void volume (ml)	1.02	0.98	0.94						

reported, as they were unaffected by the changes in operating conditions or eluent composition.

Effect of proportion of acetonitrile

The separation of the thiazide and related diuretics were then examined using different proportions of acetonitrile in the eluent (30–40%). Over this range, there were considerable changes in the capacity factors of both the alkyl aryl ketones and the diuretic drugs (Table IV, Fig. 2a). A comparison of the results for acetonitrile–1% aqueous acetic acid (30:70) in Table IV with those obtained in the initial work (Table II) emphasises the problems of interlaboratory reproducibility. Both sets of capacity factors were determined on columns packed with the same batch of packing material, but the studies were carried out in different laboratories with different HPLC systems, column sizes, and temperatures.

For each eluent composition, the logarithmic relationship between the capacity factors of the alkyl aryl ketones and their carbon numbers was linear (Fig. 3) and the slope decreased as the proportion of acetonitrile increased (Table V). Based on these values, the retention indices of the diuretic drugs were calculated (Table IV, Fig. 2b). These showed some changes with eluent composition, but the effects were much smaller than for the capacity factors. Previously, De Croo *et al.*[17] found that the capacity factors of the diuretic drugs behaved similarly as the proportion of

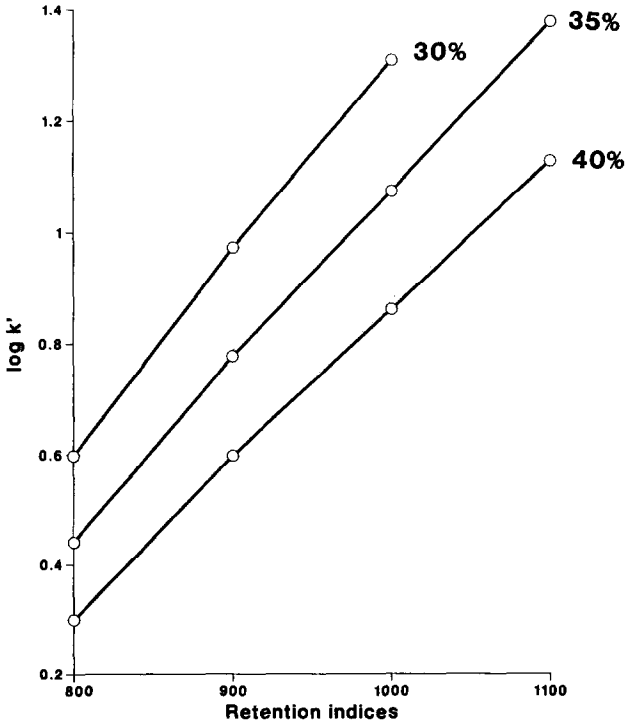


Fig. 3. Comparison of capacity factors of alkyl aryl ketones compared to their retention indices in different proportions of acetonitrile.

TABLE V

RELATIONSHIP BETWEEN THE LOGARITHM OF CAPACITY FACTORS AND NUMBER OF CARBON ATOMS FOR ALKYL ARYL KETONES IN DIFFERENT PROPORTIONS OF ACETONITRILE

Amount acetonitrile in eluent (%)	*Correlation coefficient*	*Slope* ($\times 10^3$)	*Intercept*
30	0.9995	3.56	−2.25
35	0.9996	3.11	−2.04
40	0.9996	2.75	−1.89

acetonitrile changed, with the exception of chlorthalidone. In the present study, clopamide appeared to be anomalous, as its retention index increased with the proportion of acetonitrile, whereas the retention indices for all the other compounds decreased. This probably reflects the fact that clopamide, which is basic, will be protonated under the separation conditions and thus will interact with the stationary phase in a way different from that of the other compounds.

In the earlier study on the barbiturates, relative retention times and relative capacity factors (compared to a barbiturate standard) were found to be even more robust than retention indices as a method of recording retentions[11]. It appeared that, because of the close structural similarities of the barbiturates, they would all interact in similar way with the column. If a barbiturate was selected as the internal standard, it would therefore compensate for any changes in the specific interactions in the separation. In the present study, the relative capacity factors were calculated using polythiazide as a standard (Table IV, Fig. 2c). This compound was chosen, because it has a relatively long retention, and would be less susceptible to errors in the measurement of the column void volume or in retention times. For some of the diuretics, such as benzofluazide and cyclopenthiazide, this method compensated well for the changing proportion of acetonitrile in the eluent, but for many of the other diuretics the relative capacity factors varied considerably. Even if different diuretic drugs had been considered as the standard compound, it is clear that none of them would have been able to compensate for the behaviour of all the other drugs. The same problem would have also been encountered if relative retention times had been examined, although they usually have the advantage that they can often be reported directly by many integrators.

These significant changes in the retention indices and relative capacity factors on changing the composition of acetonitrile were not unexpected, as in the preliminary experiments the retentions of the column test compounds varied considerably in this composition range[12]. However, both relative retention methods showed much smaller changes than the capacity factors. Close control over the composition of acetonitrile is therefore likely to be necessary to obtain good interlaboratory reproducibility, much more so than in the separations of the barbiturates[1] or local anaesthetics[3].

Effect of proportion of acetic acid and the pH

A series of separations were carried out in which the proportion of acetic acid

was altered from 0.7 to 1.5% (Table VI). The capacity factors and retention indices of all the compounds were virtually unaffected, except for clopamide, which showed a k' value of 4.11 at both 0.7 and 1.5% acetic acid, higher than previously measured on the same column for an eluent with 1% acetic acid ($k' = 3.64$). However, reinvestigation of the 1% acetic acid eluent also gave $k' = 4.11$. Presumably this drug must have an unusual interaction with the column or the surface properties of the packing material appear to have altered during these experiments with different concentrations of acetic acid.

Altering the proportion of acetic acid would also have had a minor effect on the pH of the eluent. However, the thiazides are weak acids (pK_a 8–10)[20] and would not be ionised in this eluent and should therefore be unaffected. In their study, De Croo *et al.*[17] found that there was no effect on the retentions of the thiazides over the range pH 3–7, but the retentions of some of the non-thiazides altered markedly. To determine whether pH was a critical factor in the present study, the pH of the aqueous solution (normally pH 2.62) was deliberately altered to pH 3.5 with sodium hydroxide or to pH 2.5 with hydrochloric acid, before the acetonitrile was added. With the exception of clopamide (pH 2.5, $RI = 849$ and pH 3.5, $RI = 706$), both the capacity factors and retention indices of all the drugs were unaffected by these

TABLE VI

EFFECT OF THE PROPORTION OF ACETIC ACID ON THE CAPACITY FACTORS OF THIAZIDE DIURETICS AND RELATED DRUGS

ODS Hypersil column (100 × 5 mm); eluent, acetonitrile–aqueous acetic acid (35:65); temperature, 30°C.

Compound	*Amount acetic acid in eluent (%)*		
	0.7	*1.0*	*1.5*
Thiazide diuretics			
Chlorothiazide	0.38	0.40	0.37
Hydrochlorothiazide	0.47	0.49	0.46
Hydroflumethiazide	0.82	0.85	0.82
Trichlormethiazide	1.68	1.72	1.67
Methyclothiazide	1.99	2.04	1.99
Benzthiazide	4.07	4.19	4.12
Cyclothiazide 1	4.64	4.78	4.69
Cyclothiazide 2	5.06	5.21	5.11
Polythiazide	6.22	6.39	6.24
Bendrofluazide	6.42	6.60	6.45
Cyclopenthiazide	6.92	7.13	7.04
Non-thiazide diuretic drugs			
Quinethazone	0.47	0.46	0.54
Chlorthalidone	0.71	0.76	0.72
Metolazone	2.46	2.54	2.50
Clorexolone	3.51	3.63	3.63
Mefruside	4.30	4.43	4.37
Clopamide	4.10	3.64*	4.11
Column void volume (ml)	0.98	0.98	0.98

* On repeating separation with 1.0% acetic acid $k' = 4.11$ (see Discussion).

changes. The proportion of acetic acid and the pH are clearly not critical factors in this assay, except for the basic diuretic, clopamide.

Effect of temperature

The temperature of the column was altered from 10–40°C, and the capacity factors, retention indices, and relative capacity factors of the drugs and column test compounds were calculated (Table VII). All compounds showed a marked decrease in their capacity factors with increasing temperature and an increase in efficiency, *e.g.* polythiazide $N = 554$ at 10°C and $N = 2128$ at 40°C. In a similar study over the range 25–50°C, De Croo *et al.*[17] found that the thiazide drugs gave a linear relationship between their capacity factors and the reciprocal of the absolute temperature (Van 't Hoff plots).

The retention indices showed much smaller variations and, over a limited temperature range, would be effectively constant. The relative capacity factors changed to different extents, depending on the compound. These results again confirm the advantages of relative retention values over direct measurements. If the columns are nominally controlled at the same temperature in different laboratories, any small differences in temperature which may occur are unlikely to have a significant effect on relative capacity factors or retention indices but might still have a marked effect on absolute capacity factors.

On comparing the results from the initial study at ambient temperature (Table II) with the values at 30°C for the same composition (Table IV), both the capacity factors and relative capacity factors were found to differ, confirming that, for identification purposes, temperature control is important for this HPLC separation.

Reproducibility of repeated measurements

The separations under the standard conditions [acetonitrile–1% aqueous acetic acid (35:65)] with the same column and equipment were repeated daily for four days, using fresh preparations of eluent, and were compared with the initial chromatogram to test the reproducibility. The mean values, standard deviations (S.D.), and coefficients of variance (C.V.) for the capacity factors and retention indices were calculated (Table VIII). For almost all the samples, the results from the different runs were very close with variations similar to those obtained in earlier drug separation studies[1,3], showing that high reproducibility can be obtained if the conditions are carefully controlled. However, the capacity factors of clopamide and quinethazone had changed since the first analysis. Although the repeated separations on successive days gave consistent values these were markedly different from the first measurement. Apparently during the study with different pH eluents the nature of the column surface had been altered.

Effect of the stationary phase

Differences in the retentions of compounds on nominally equivalent stationary phases, such as ODS-silicas, are probably the greatest source of differences between separations reported in the literature. Major differences in the properties of bonded-phase columns can occur, even if they are coated with the same alkyl chains, because different manufacturers use different silicas and different bonding methods. Furthermore, batch-to-batch variations in the efficiency of the bonding reactions and capping

TABLE VII

EFFECT OF TEMPERATURE ON THE CAPACITY FACTORS, RETENTION INDICES, AND RELATIVE CAPACITY FACTORS OF ALKYL ARYL KETONES, COLUMN TEST COMPOUNDS, THIAZIDE DIURETICS, AND RELATED DRUGS

ODS Hypersil column (100 × 5 mm); eluent, acetonitrile–1% aqueous acetic acid (35:65).

Compound	*Capacity factor*				*Retention index*				*Relative capacity factor × 100*			
	Temperature (°C)				*Temperature (°C)*				*Temperature (°C)*			
	10	*20*	*30*	*40*	*10*	*20*	*30*	*40*	*10*	*20*	*30*	*40*
Retention index standards												
Acetophenone	3.67	3.37	2.76	2.50								
Propiophenone	8.26	7.57	6.00	5.32								
Butyrophenone	17.05	15.56	11.89	10.39								
Valerophenone	36.71	33.21	24.13	20.76								
Column test compounds												
2-Phenylethanol	1.73	1.69	1.45	1.35	699	706	706	709				
p-Cresol	3.20	2.89	2.30	2.04	780	777	771	768				
Nitrobenzene	6.90	6.04	4.68	4.00	880	875	870	864				
Methyl benzoate	7.35	6.72	5.31	4.70	889	889	888	887				
Toluene	18.49	16.69	12.72	11.00	1009	1008	1009	1009				

Thiazide diuretics												
Chlorothiazide	0.65	0.52	0.40	0.32	571	551	527	504	5.3	5.3	6.3	6.4
Hydrochlorothiazide	0.80	0.64	0.49	0.40	598	578	555	536	6.5	6.5	7.7	8.0
Hydroflumethiazide	1.47	1.15	0.85	0.68	678	656	632	611	12.0	11.6	13.3	13.6
Trichlormethiazide	3.02	2.41	1.72	1.38	772	753	730	712	24.6	24.3	26.9	27.5
Methyclothiazide	3.61	2.89	2.04	1.64	796	777	754	737	29.4	29.2	31.9	32.7
Benzthiazide	7.71	6.26	4.19	3.34	895	879	855	838	62.8	63.2	65.6	66.5
Cyclothiazide 1	8.39	7.01	4.78	3.88	906	894	873	859	68.4	70.8	74.8	77.3
Cyclothiazide 2	9.37	7.75	5.21	4.20	920	907	885	871	76.4	78.3	81.5	83.7
Polythiazide	12.27	9.90	6.39	5.02	956	940	913	896	100.0	100.0	100.0	100.0
Bendrofluazide	12.29	10.29	6.60	5.14	963	945	918	899	105.8	103.9	103.3	102.4
Cyclopenthiazide	12.82	10.70	7.13	5.73	961	950	929	915	104.5	108.1	111.6	114.1
Non-thiazide diuretic drugs												
Quinethazone	0.61	0.55	0.46	0.40	563	558	546	536	5.0	5.6	7.2	8.0
Chlorthalidone	1.04	0.94	0.76	0.66	633	629	616	607	8.5	9.5	11.9	13.1
Metolazone	3.78	3.37	2.54	2.20	802	797	785	779	30.8	34.0	39.8	43.8
Clorexolone	4.48	4.66	3.63	3.29	835	840	834	836	36.5	47.1	56.8	65.5
Mefruside	6.73	5.98	4.43	3.80	877	893	862	856	54.8	60.4	69.3	75.7
Clopamide	5.51	4.40	3.64	3.11	851	833	835	828	44.9	44.4	57.0	61.9
Column void volume (ml)	0.98	0.98	0.98	0.98								

TABLE VIII

REPRODUCIBILITY OF THE CAPACITY FACTORS AND RETENTION INDICES OF THIAZIDE DIURETICS AND RELATED DRUGS

ODS Hypersil column (100 × 5 mm); eluent, acetonitrile–1% aqueous acetic acid (35:65); temperature, 30°C. Based on five separate determinations on different days on the same systems with fresh eluent.

Compound	*Capacity factor*			*Retention index*		
	Mean	*S.D.*	*C.V. (%)*	*Mean*	*S.D.*	*C.V. (%)*
Thiazide diuretics						
Chlorothiazide	0.40	0.01	2.5	526	2.8	0.53
Hydrochlorothiazide	0.48	0.01	2.1	552	1.8	0.33
Hydroflumethiazide	0.83	0.01	1.2	630	1.3	0.21
Trichlormethiazide	1.68	0.02	1.2	729	1.1	0.15
Methyclothiazide	2.00	0.03	1.5	753	1.3	0.17
Benzthiazide	4.07	0.08	2.0	853	1.8	0.21
Cyclothiazide 1	4.65	0.09	1.9	871	1.1	0.13
Cyclothiazide 2	5.06	0.10	2.0	883	1.2	0.14
Polythiazide	6.19	0.14	2.3	911	1.2	0.13
Bendrofluazide	6.41	0.14	2.2	916	1.2	0.13
Cyclopenthiazide	6.92	0.16	2.3	927	1.5	0.16
Non-thiazide diuretic drugs						
Quinethazone*	0.57	0.01	1.8	578	3.0	0.52
Chlorthalidone	0.74	0.02	2.7	613	2.3	0.38
Metolazone	2.48	0.04	1.6	783	1.5	0.19
Chlorexolone	3.54	0.06	1.7	833	1.1	0.13
Mefruside	4.32	0.08	1.9	861	1.1	0.13
Clopamide*	4.73	0.10	2.1	874	4.0	0.46

* Based on four measurements on consecutive days. Fifth value measured on same column two weeks earlier; quinethazone, $k' = 0.46$, $RI = 546$; clopamide, $k' = 3.64$, $RI = 835$.

steps by a single manufacturer are also significant. Even larger changes are found, if different alkyl bonded groups are used. De Croo *et al.*[18] compared the retentions of RP-2, RP-8, and RP-18 Lichrosorb and found that, although the thiazides gave fairly similar results, large differences were seen for the non-thiazide diuretic drugs.

The separation of the drugs was therefore repeated, using a range of different ODS-silica packing materials. Two different batches of ODS-Hypersil were also included in the comparison. In the study of the separation of barbiturates, negligible differences were found between batches of this packing material[2], and virtually identical results were also obtained in this study (Table IX). However, considerable variations in the capacity factors were observed for both the diuretic drugs and the column test compounds on the other brands of packing material.

The retention indices were calculated for each column, but the results have a greater spread than would be useful for identification purposes (Table X). The ODS-Zorbax column gave particularly unusual results with much smaller retention indices that the other columns. This effect was reflected also by the column test compounds on this column, particularly the low retention index value for *p*-cresol. Similar results with this column material were previously noted in column comparison studies[9].

TABLE IX

EFFECT OF ODS-SILICA COLUMN PACKING MATERIAL ON THE CAPACITY FACTORS OF ALKYL ARYL KETONES, COLUMN TEST COMPOUNDS, THIAZIDE DIURETICS, AND RELATED DRUGS

Columns, 100 × 5 mm; eluent, acetonitrile–1% aqueous acetic acid (35:65); temperature, 30°C. Columns: H1, ODS-Hypersil Batch 10/1229; H2, ODS-Hypersil Batch 6/868; Z, ODS-Zorbax; T, Techsil 5 C18; TS; Techsphere ODS; L, Lichrosorb RP 18; N, Nucleosil 5 C18.

Compound	*Capacity factor*								
	Column material							*Mean*	*S.D.*
	H1	*H2*	*Z*	*T*	*TS*	*L*	*N*		
Retention index standards									
Acetophenone	2.76	2.78	5.11	2.67	3.58	3.57	3.80	3.47	0.86
Propiophenone	6.00	5.94	11.07	5.51	7.84	7.56	8.02	7.42	1.90
Butyrophenone	11.89	11.68	22.18	10.42	15.66	14.65	15.50	14.57	3.93
Valerophenone	24.13	23.67	45.86	20.33	32.17	29.34	30.83	29.48	8.40
Column test compounds									
2-Phenylethanol	1.45	1.50	2.34	1.40	1.74	1.91	1.93	1.75	0.34
p-Cresol	2.30	2.36	3.32	2.13	2.78	2.94	3.03	2.69	0.44
Nitrobenzene	4.68	4.66	8.18	4.51	6.08	6.00	6.54	5.81	1.33
Methyl benzoate	5.31	5.22	9.92	4.95	6.97	6.81	7.20	6.63	1.72
Toluene	12.72	11.81	23.65	10.71	17.37	15.63	16.19	15.44	4.37
Thiazide diuretics									
Chlorothiazide	0.40	0.43	0.41	0.38	0.43	0.48	0.52	0.44	0.05
Hydrochlorothiazide	0.49	0.53	0.53	0.46	0.52	0.60	0.64	0.54	0.06
Hydroflumethiazide	0.85	0.91	1.00	0.83	0.96	1.05	1.16	0.97	0.12
Trichlormethiazide	1.72	1.90	2.17	1.67	1.97	2.14	2.41	2.00	0.26
Methyclothiazide	2.04	2.27	2.63	2.00	2.33	2.55	2.87	2.38	0.32
Benthiazide	4.19	4.72	5.40	3.88	4.75	5.17	5.72	4.83	0.65
Cyclothiazide 1	4.78	5.38	6.17	4.31	5.39	6.29*	6.36	5.40	0.79
Cyclothiazide 2	5.21	5.82	6.74	4.64	5.88	6.29*	6.87	5.92	0.80
Polythiazide	6.39	7.04	8.59	5.91	7.44	7.75	8.81	7.42	1.07
Bendrofluazide	6.60	7.26	9.08	6.03	7.81	7.98	8.99	7.68	1.14
Cyclopenthiazide	7.13	7.91	9.08	6.16	8.04	8.49	9.14	7.99	1.07
Non-thiazide diuretic drugs									
Quinethazone	0.46	0.46	0.48	0.44	0.48	0.55	0.59	0.49	0.05
Chlorthalidone	0.76	0.84	0.80	0.71	0.79	0.92	0.99	0.83	0.10
Metolazone	2.54	2.80	3.16	2.32	2.81	3.09	3.35	2.87	0.36
Chlorexolone	3.63	4.03	4.58	3.13	3.93	4.34	4.54	4.03	0.52
Mefruside	4.43	4.80	6.07	3.98	5.16	5.30	5.82	5.08	0.74
Clopamide	3.64	3.21	6.12	1.41	4.43	3.04	1.68	3.36	1.61
Column void volume	0.98	1.10	0.82	1.12	0.88	1.10	1.10		

* Only one peak observed on this column.

When the relative capacity factors of the thiazide drugs were calculated, they gave better correlations between the different columns and virtually compensated for the unusual behaviour of ODS-Zorbax (Table XI). This is in contrast to the earlier comparisons when relative capacity factors did not compensate for differences in the mobile phase or the temperature. Irrespective of the method of recording retentions,

TABLE X

EFFECT OF ODS-SILICA COLUMN PACKING MATERIAL ON THE RETENTION INDICES OF COLUMN TEST COMPOUNDS, THIAZIDE DIURETICS, AND RELATED DRUGS

Separation conditions and columns as Table IX.

Compound	*Retention index*								
	Column material							*Mean*	*S.D.*
	H1	*H2*	*Z*	*T*	*TS*	*L*	*N*		
Column test compounds									
2-Phenylethanol	706	707	690	701	698	708	699	701	6.4
p-Cresol	771	771	739	763	762	769	764	763	11.1
Nitrobenzene	870	868	862	875	870	872	875	870	4.5
Methyl benzoate	888	884	889	889	889	890	889	888	1.9
Toluene	1009	1001	1008	1003	1014	1009	1006	1007	4.3
Thiazide diuretics									
Chlorothiazide	527	529	451	506	506	510	511	506	25.9
Hydrochlorothiazide	555	559	486	535	532	542	540	536	24.0
Hydroflumethiazide	632	636	574	623	616	622	626	618	20.7
Trichloromethiazide	730	741	680	727	715	724	732	721	19.8
Methyclothiazide	754	766	706	754	738	749	756	746	19.6
Benzthiazide	855	870	805	852	836	850	856	846	20.8
Cyclothiazide 1	873	889	824	868	853	878	871	865	21.1
Cyclothiazide 2	885	900	836	879	865	878	882	875	20.1
Polythiazide	913	927	869	915	897	908	918	907	19.0
Bendrofluazide	918	931	877	918	904	912	921	912	17.4
Cyclopenthiazide	929	944	877	921	908	921	923	918	20.9
Non-thiazide diuretic drugs									
Quinethazone	546	539	473	529	521	529	528	524	23.7
Chlorthalidone	616	625	543	600	589	603	603	597	26.5
Metolazone	785	796	732	776	764	776	779	773	20.3
Chlorexolone	834	848	783	821	810	825	823	821	20.3
Mefruside	862	873	821	856	847	854	858	853	16.2
Clopamide	835	815	822	702	826	774	679	779	63.8

clopamide showed major differences between the various columns, presumably reflecting its partial ionisation and different interaction.

CONCLUSIONS

Clearly, the use of the present HPLC system for the identification of the diuretic drugs requires certain aspects of the experimental conditions to be closely defined, if good interlaboratory reproducibility is to be obtained. In particular, the proportion of acetonitrile in the mobile phase, the column temperature, and the brand of column packing material are important. A common brand of packing material should be adopted for interlaboratory comparisons. However, the pH and the exact proportion of acetic acid in the aqueous component are not critical, except for a few non-thiazide analytes.

TABLE XI

EFFECT OF ODS-SILICA COLUMN PACKING MATERIAL ON THE RELATIVE CAPACITY FACTORS OF THIAZIDE DIURETICS AND RELATED DRUGS

Separation conditions and columns as Table IX.

Column	*Relative capacity factor* × *100*								
	Column material							*Mean*	*S.D.*
	H1	*H2*	*Z*	*T*	*TS*	*L*	*N*		
Thiazide diuretics									
Chlorothiazide	6.2	6.1	4.8	6.4	5.8	6.2	5.9	5.9	0.6
Hydrochlorothiazide	7.7	7.5	6.2	7.8	7.0	7.7	7.3	7.3	0.6
Hydroflumethiazide	13.3	12.9	11.6	14.0	12.9	13.6	13.2	13.1	0.7
Trichlormethiazide	26.9	27.0	26.3	28.3	26.5	27.6	27.4	27.0	0.9
Methyclothiazide	31.9	32.2	30.6	33.8	31.2	32.9	32.6	32.2	1.0
Benzthiazide	65.6	67.0	62.8	65.6	63.8	66.7	64.9	65.2	1.5
Cyclothiazide 1	74.8	76.4	71.8	72.9	72.4	81.2	72.2	74.5	3.3
Cyclothiazide 2	81.5	82.7	78.5	78.5	79.0	81.2	78.0	79.9	1.8
Bendrofluazide	103.3	103.1	105.7	102.0	105.0	103.0	102.0	103.5	1.4
Cyclopenthiazide	111.6	112.4	105.7	104.2	108.1	109.6	103.8	107.9	3.5
Non-thiazide diuretic drugs									
Quinethazone	7.2	6.5	5.6	7.4	6.4	7.1	6.7	6.7	0.6
Chlorthalidone	11.9	11.9	9.3	12.0	10.6	11.9	11.2	11.3	1.0
Metolazone	39.7	39.8	36.8	39.3	37.8	39.9	38.0	38.7	1.2
Clorexolone	56.8	57.2	53.3	52.9	52.8	56.0	51.5	54.4	2.3
Mefruside	69.3	68.2	70.7	67.3	69.4	68.4	66.1	68.5	1.5
Clopamide	57.0	45.6	71.1	23.9	59.5	39.2	19.1	45.1	19.1

It is important to use a relative retention method, such as relative retention times, relative capacity factors, or retention indices to record the results, but there is no clear advantage of one method over the other, as none of the diuretic drugs appear to be ideal as a standard. Relative capacity factors appear to have an advantage when different column materials are being used, although significant differences are still present, which would reduce the reliability of any identification. Retention indices have the advantage that a common scale can be used for different HPLC systems, and in our study the method proved to be virtually as robust as relative capacity factors.

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