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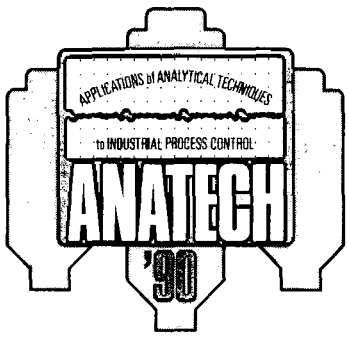

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Noordwijkerhout, The Netherlands, 3-5 April 1990

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Symposium Secretariat ANATECH '90
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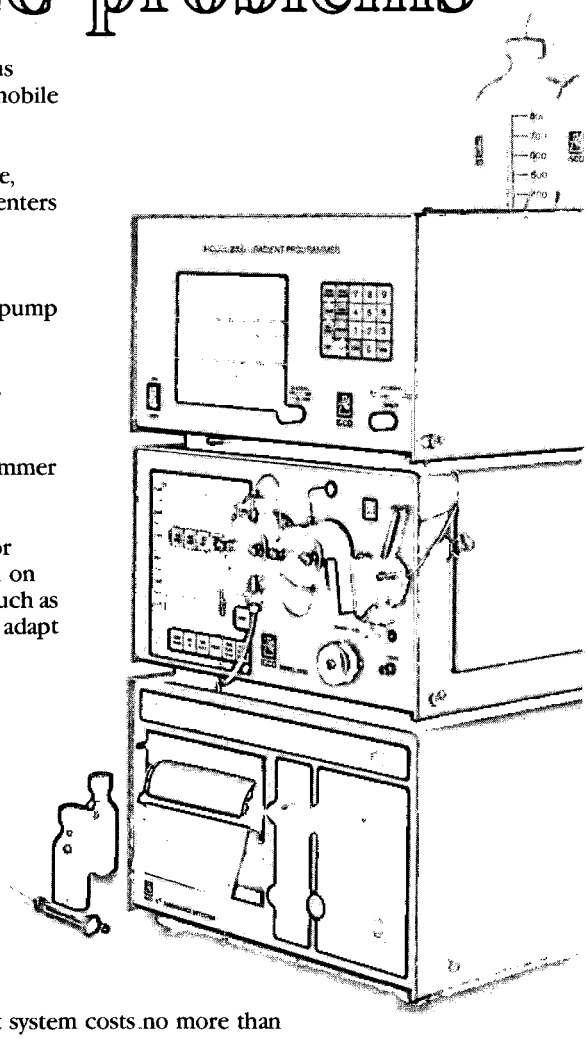

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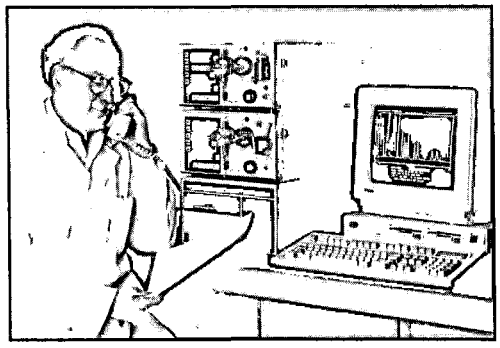

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REVIEW

THIRTIETH ANNIVERSARY OF THE RETENTION INDEX ACCORDING TO KOVÁTS IN GAS–LIQUID CHROMATOGRAPHY

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1. INTRODUCTION

"Theory (when correct) and experiment (if carefully executed) describe the same truths".

J. Calvin Giddings[446]

As pointed out in our previous review dealing with the retention index system[47], the Kováts retention index[434], introduced in 1958, turned out to be perhaps the most significant contribution to the theory and practice of gas–liquid chromatography (GLC) since the pioneering discovery of the method by James and Martin in 1952[444].

Whereas the instrumental basis, technical level and capacity of GLC have reached the desired levels during about the last four decades, this is not so, unfortunately, with the retention index system. Although some researchers have obtained important results in this field, the badly needed decisive breakthrough has not yet occurred. In fact, only about one quarter of gas chromatographic workers are familiar with the retention index system and only about 10% of analytical chemists have adopted it for everyday use in research work, although current estimates show that about 10^5 gas chromatographs are being operated worldwide by nearly 250 000 experts.

The Kováts retention index is the only retention value in GLC in which the two fundamental quantities, *viz.*, the relative retention and the specific retention volume, are united[445]. Moreover, a series of explicit relationships between retention indices and the physico-chemical quantities related to GLC have been developed. Similarly, numerous linear relationships between the retention index and other fundamental quantities such as boiling point, carbon number, molecular weight and molar refraction have been derived.

Published retention data are given in many forms, and therefore their usefulness in different laboratories is limited. The Kováts retention index[434] solves the problem of the uniform expression of retention data.

Regarding the prevalence of the retention index concept, the first half of the 1980s were important years. The number of workers in gas chromatography interested in this topic grew over the years and important new research centres investigating the retention index system were set up[1–3,5,8,28,34,37,42,45,60,69,72,78,142,154,162,187,210,212,265,354].

An important year in the development and spreading of the retention index system was 1983, the Tenth Hungarian Chromatographic Symposium entitled the Twenty-Fifth Anniversary of the Retention Index System being held in Sárospatak (Hungary). Professor Kováts (Institute of Physical Chemistry, École Polytechnique, Lausanne, Switzerland) was the Chairman of the Retention Index Symposium. His opening lecture and further lectures dealt with the theoretical and practical questions of the retention index system[176,184,221,226,227,229,230,233,234]. These lectures and the subsequent discussions considerably promoted the further development of retention index system.

Numerous researchers in the second half of the 1980s have worked on various aspects of the retention index system, including the determination of the dead time, retention index determinations in temperature-programmed GC and the connection between retention index and molecular structure, and interesting results were published in nearly 200 papers[241–433]. This progress, although its rate was slower than in the past, is characteristic of the end 1980s.

Obviously, in this review we are unable to give a full account of the activities of the hundreds of researchers who have achieved important results in this field. Without

aiming at completeness, the following and their co-workers have published numerous papers: Bata[1,41,97,147], Berezkin[2a,98,99,163,268], Bermejo[3,42,101,102,165–167,247,323–327], Boneva[5,43,44,249–253,259,329,330,407], Brooks[45,46,102a,331,402,403], Buryan[164,254,332,461], Calixto[8,9,51,51a,52,104,104a,138,170a], Castello[10,10a,10b,53,105,257,257a,404], Churácek[182,275,276,281,473], Cole[45,46,102a,331,402,403], Dimov[5,43,44,173,249,250,259–261,330,335,407], Fernandez[2,142,216–218,306,377–379], Franke[34,42a,168,265,266,328,344], García-Dominguez[2,142,216–218,306,377–379], García-Raso[8,9,51,51a,52,104,104a,138,170a,212a,212b,300,374,375], Golovnya[14,58,58a,58b,109a–d,174a,175a,175b,225a–c,267a,267b,268a,280a,290a,298a,305a,309a,347,347a,348,348a,411,460,470], Guillen[42,101,102,165–167,247,323–327], Haken[60–65,111–113,170b,177–180,269–271,469], Kersten[354,355,370,371,424], Korhonen[60–63,111,112,119–125,177–180,185–196,269,270,282–284], Lakszner[212,212a,227,298], Lamparczyk[72,73,126,200,287,287a,359a], Lind[187–190,193,194], Macák[164,254,332,461], Madden[60,64,113,180], Menendez[142,216,218,306,377–379], Molera[2,217,218,377,379,459], Munoz[2,217,218,377,379], Nabivach[69,164,254,322a,332,365,461,463], Osek[210,292,293,295,296], Oszczapowicz[210,292,293,295,296], Podmaniczky[212,212a,227,298], Pomaville[369–371,423], C. F. Poole[28,135,354,355,370,371,423,424,424a], S. K. Poole[370,371,424,424a], Radecki[72,73,126,287a], Rang[27,32,142b,268,373], Rimada[142,216,217,377–379], Soják[2a,35b,35c,142b,142c,142d,309,381,382,428,429,477], Szepesy[212,212a,226,227,298], Szymanowski[37,162,228,310,384], Vernon[64,88,89,271], Voelkel[162,228,310,387–389,432], Wainwright[90,222–224], T. Wang[235,392–395], F. Wang[154,234a,313,390,391], Wijsbeek[34,168,265,266,344] and De Zeeuw[168,265,266,328,344].

During research work carried out in the last 5 years, striking results have been obtained by individual researchers that have had a major influence on progress in studies of the retention index system. Without trying to be exhaustive, the research work of Albaiges, Berlizov, De Bertoli, Bonastre, Chien, Chretien, Cramers, Czerwiec, D'Agostino, D'Amato, Eglinton, Engewald, Eisen, Ellren, Ettre, Evans, García-Raso, Grenier, Guermouche, Hawkes, Janák, Jaworski, Kaliszan, Komarek, Kováts, Krupcik, Kuningas, Lee, Mihara, Orav, Pacákova, Papazova, Peetre, Rijks, Schomburg, Singliar, Tesarik, Torres, Tóth, Vigdergauz, Wu and Zhuravleva should be mentioned.

In the following, the main theoretical and practical questions concerning the retention index system will be summarized. First, the nomenclature and the most important definitions and relationships will be recapitulated.

2. NOMENCLATURE, SYMBOLS AND DEFINITIONS

During the last 5 years, about 500 papers have been published in this field of GLC and identical symbols were mostly used throughout. The uniform system of symbols of relating to retention indices was summarized in our previous review[47]. As pointed out by Ryba[435], the retention index is a dimensionless quantity. However, for the sake of a simpler discussion, "retention index units" (i.u.) were introduced. For similar reasons, retention index units are also considered to have "dimensions" in the case of structural index increments, ΔI values, etc.

The basic equation of the retention index elaborated by Kováts[434] and the various equations derived from this equation using equivalent mathematical transformations[a] are the most frequently discussed aspect of the retention index system and

[a] The term "equivalent mathematical transformation" means that in the equation for the retention index (*e.g.*, eqn. 2 in ref. 47) the adjusted retention time (t'_R) is used rather than the specific retention volume (V_g).

have remained in the centre of interest of various investigators and research groups. Of the numerous outstanding results that have been published, those of Smith, Haken and Wainwright[223], Belyaev and Vigdergauz[245], Evans, Haken and Tóth[264], Heeg and Zinburg[274], Dose[338] and Gusev, Rang, Berezkin and Orav[268] should be mentioned.

Thermodynamic characteristics determined by Podmaniczky *et al.*[212a] from isothermal retention data were used for retention index calculations. The minimum retention index difference required for the identification of two adjacent compounds and the temperature dependence of retention index were discussed.

Several statistical and iterative methods for the calculations of the retention indices were compared by Smith *et al.*[222] using retention data obtained on a wide range of columns (SE-30, Apiezon-L, SF-96, OV-17, OV-25, etc.) at different column temperatures.

Electric interaction indices for a series of twelve monomethylbenz[*a*]anthracenes were calculated by Lamparczyk and Ochocka[287] from GC relative retention times, using anthracene and benz[*a*]anthracene as reference compounds. Application of these electric interaction indices for structure–biological activity studies and the role of electric interactions in the retention index concept were discussed.

An interesting general model consisting of two terms was proposed by Dimov[335] for the calculation of the retention indices of isoalkanes on squalane as stationary phase. The first term (referred to as extensive) includes parameters that have the greatest correlation with the experimental value of the retention index. The second term (referred to as intensive) includes parameters that can modify the value of the roughly calculated retention index in the direction of the experimental value. The equations derived by Dimov[335] had correlation coefficients better then 0.99.

Some of the results relating to the above are given in Tables 1–6, without claiming to be comprehensive. For further details, see refs. 2a, 28a, 34a, 35a, 35b, 35c, 35d, 54, 55, 55a, 58a, 58b, 82, 90, 93a, 96, 105, 105b, 118a, 126a, 142a, 142b, 142c, 142d, 170b, 173, 198a, 200, 212b, 216, 217, 225b, 225c, 229, 233a, 251, 257a, 258a, 259, 267a, 267b, 268a, 274, 277, 287a, 290a, 298a, 305a, 307, 309a, 310, 311, 318a, 322a, 325, 341, 346a, 347a, 348a, 362, 379a, 386a, 401, 410a, 411a, 429b, 457 and 469.

TABLE 1

COMPARISON OF RETENTION INDICES CALCULATED BY BALLSCHMITER *ET AL.*[161] USING DIFFERENT CALCULATION METHODS

Carbon No.	*Calculated retention index (i.u.)*		
	Classical method	*x^3 polynomial method*	*x^4 polynomial method*
14	1399.04	1399.75	1399.96
15	1498.57	1500.02	1500.03
16	1598.63	1600.11	1600.05
17	1698.99	1700.00	1699.93
18	1800.00	1800.22	1800.18

TABLE 2

MOLECULAR STRUCTURAL COEFFICIENTS OF SELECTED COMPOUNDS ON APOLANE-87 STATIONARY PHASE AT 70°C

Compound	*Structural coefficient*	*Compound*	*Structural coefficient*
Cyclopentane	482.61	1-Chloroheptane	841.21
Cyclohexane	582.31	1-Bromopropane	416.31
Cycloheptane	718.31	1-Bromobutane	520.91
1-Pentyne	391.21	1-Bromopentane	623.51
1-Hexyne	494.01	1-Bromohexane	724.41
1-Heptyne	593.81	1-Bromoheptane	825.01
1-Octyne	692.51	Iodomethane	435.31
1-Nonyne	792.01	Iodoethane	524.31
Benzene	565.91	1-Iodopropane	627.01
Toluene	673.51	1-Iodobutane	726.01
Ethylbenzene	761.91	1-Iodopentane	824.51
n-Propylbenzene	849.71	1-Butanol	508.91
1-Chloropropane	433.61	1-Pentanol	607.01
1-Chlorobutane	537.11	1-Hexanol	704.71
1-Chloropentane	638.91	1-Heptanol	805.01
1-Chlorohexane	740.01		

TABLE 3

MOLECULAR STRUCTURAL COEFFICIENT OF SELECTED HYDROCARBONS MEASURED BY CHIEN *ET AL.*[54] ON OV-101 STATIONARY PHASE AT DIFFERENT COLUMN TEMPERATURES

Compound	*Structural coefficient*[a]			
	30°C	*50°C*	*70°C*	*90°C*
n-Pentane	421.79	402.70	381.28	357.15
n-Hexane	521.87	502.73	481.25	457.07
n-Heptane	621.82	602.70	581.23	557.04
n-Octane	721.76	702.72	681.22	656.87
n-Nonane	821.83	802.71	781.21	756.97
n-Decane	921.84	902.68	881.17	856.92
cis-2-Pentene	437.06	417.60	395.73	371.05
2,2-Dimethylbutane	455.81	438.12	418.32	396.08
Benzene	569.02	553.85	536.81	517.61

[a] Value calculated by Antoine equation:

$$S_{c\ \text{substance}}^{\text{OV-101}}(T) = a^* + \frac{b^*}{T + c^*} \qquad b^* \qquad (1)$$

where S_c is the molecular structural coefficient, T is the column temperature (K) and a^*, b^* and c^* are constants in eqn. 1. For example, for benzene $a^* = -2.8458$, $b^* = 1603.8490$ and $c^* = -0.8747$.

TABLE 4

KOVÁTS COEFFICIENTS ON SQUALANE STATIONARY PHASE AT DIFFERENT COLUMN TEMPERATURES

Carrier gas, nitrogen.

Column temperature (°C)	*Kováts coefficient*	*Column temperature (°C)*	*Kováts coefficient*
30	71.43	80	108.70
40	78.19	90	117.32
50	85.28	100	126.40
60	92.71	110	135.98
70	100.51	120	146.10

TABLE 5

KOVÁTS COEFFICIENTS ON SE-30 AND APOLANE-87 STATIONARY PHASES AT DIFFERENT COLUMN TEMPERATURES

Carrier gas, helium.

Column temperature (°C)	*Kováts coefficient*	
	SE-30	*Apolane-87*
30	66.36	73.71
40	75.96	80.39
50	86.00	86.92
60	97.10	93.29
70	108.30	99.52
80	120.30	105.60

TABLE 6

KOVÁTS COEFFICIENTS ON SOME OV STATIONARY PHASES AT 80°C

Carrier gas, helium. Retention data used for calculations are from Chien *et al.*[54,55].

Stationary phase	*Phenyl (%)*	*Kováts coefficient*
OV-1	0	117.49
OV-101	0	130.18
OV-3	10	149.10
OV-7	20	166.47
OV-61	33	187.04
OV-11	35	190.02
OV-17	50	211.00
OV-22	65	229.81
OV-25	75	241.30

3. RETENTION INDICES IN PROGRAMMED TECHNIQUES IN GLC

In order to facilitate the analytical application of GLC to the investigation of samples with wide boiling point ranges (most natural samples belong to this group), programmed techniques were developed, involving temperature, flow and double temperature and flow programming[448–450].

Under the conditions of pressure- and/or flow-programmed GLC, the retention index will remain constant and identical with the value measured without programming, as the carrier gas influences only the calculation of the net retention volumes and not the retention indices[47].

The widespread use of personal and home computers in the laboratory has provided the possibility of calculating double programmed retention indices, but the progress is far from satisfactory. The problem is not connected with the computer software, but with a lack of reliable GC data to be used in calculations.

Temperature-programmed GLC is one of the most important techniques and about 75% of GLC analyses are carried out under these conditions. The main problem is connected with the reproducibility of the retention indices under different laboratory conditions. Only part of the difficulties can be attributed to a lack of reliable data or to the discrepancy between the various methods for retention index calculations. The basic problem is due to the wide variety of carrier gas systems, column temperature control units and other regulation systems used in gas chromatographs of different types. In addition, column used influences the elution temperature. Although in this review we cannot discuss all these problems in detail, relevant publications are cited.

Consider the use of a linear temperature programme with a constant heating rate during the whole analysis, starting at the instant of sample introduction, so that all components are eluted during this programme. Under such conditions (classical temperature programming), the temperature-programmed retention index (I_{TPGC} or I_{TP}) can be calculated from the equation of Van den Dool and Kratz[47]. The calculation of the temperature-programmed retention index when temperature programming is combined with isothermal periods or where the programming rate is changed during the analysis (I_{cTPGC} or I_{cTP}) is more complicated[436].

In order to study elution temperatures, the interactions on Apolane-87 and OV-101 at different column temperatures were investigated by Borwitzky and Schomburg[6]. A method based on polynomial interpolation for calculating linear temperature-programmed retention indices was described by Knoeppel *et al.*[20,21]. The determination of linear temperature-programmed retention indices and the identification of phenols were discussed by White and Li[39a].

More than 180 peaks were identified and their corresponding temperature-programmed retention indices determined by Wu and Lu[93a]. The retention indices obtained varied with the GC conditions such as column length, film thickness and heating rate. A short overview of some of the most important papers dealing with the determination of temperature-programmed retention indices was given by Szepesy *et al.*[227].

Akporhonor *et al.*[241] dealt with the conversion of retention indices into those under temperature-programmed conditions. A method was presented by Podmaniczky *et al.*[298] for the calculation of retention indices in linear temperature-programmed GLC on the basis of isothermal retention data and the operating conditions (initial temperature, heating rate, etc.) of the run.

Interesting mathematical procedures were described by Akporhonor *et al.*[318a] for the calculation of temperature-programmed retention indices. A new method was presented by Chen *et al.*[319] for the calculation of the retention indices under linear temperature programming conditions with or without an initial isothermal period.

A simple procedure was demonstrated by Krupčik *et al.*[358] for the prediction of temperature-programmed retention indices from isothermal retention indices. The effect of the solute sample size on the measurement of temperature-programmed retention indices was studied by Wang and Sun[392]. Temperature-programmed retention indices calculated according to three different definitions were compared by Wang and Sun[394]. The advantages and shortcomings of these definitions were discussed in terms of their theoretical background, accuracy, ease of calculation and applicability. A broad research programme was carried out by the same group[395] using various compounds as model substances.

Summarizing this theme, some interesting results are presented in Tables 7–25. For further information about temperature-programmed retention indices, see also refs. 16, 20, 21, 24, 38, 132, 142a, 157, 171, 172, 235, 239, 240, 262, 302, 305, 380 and 393.

4. RETENTION INDEX AND GAS CHROMATOGRAPHIC PARAMETERS

The retention index depends on the chemical nature of the substance examined, on the chemical nature of the stationary phase and on the column temperature[47,434]. The most important requirement is to know the exact quality and amount (retention index calculation without specific retention volume) of the stationary phase used and their effects on the value of the retention index. Whereas it is true that the effect of the amount of the stationary phase can be eliminated by using the specific retention volume (V_g) in the retention index calculations, the quality differences between

TABLE 7

COMPARISON OF RETENTION INDICES (i.u.) MEASURED BY KRUPČIK *ET AL.*[358] UNDER CONDITIONS OF TPGC WITH DIFFERENT HEATING RATES

Initial temperature, 50°C; stationary phase, squalane.

Compound[a]	*Heating rate (°C/min)*			
	1	*2*	*3*	*5*
2,3,5-M_3C_6	812.0	813.1	813.6	814.7
2,3-M_2C_7	848.6	850.2	851.2	852.4
3,3-E_2C_5	867.0	869.5	871.2	873.7
Isopropylbenzene	906.3	907.7	908.6	910.2
4,4-M_2C_8	916.6	918.0	918.8	919.9
1,3,5-M_3-benzene	945.8	948.5	950.4	952.7
sec.-Butylcyclo-C_6	1012.8	1015.3	1017.2	1020.0
n-Butylcyclo-C_6	1020.4	1023.0	1024.8	1027.3
1,2-E_2-benzene	1036.1	1039.3	1041.4	1044.5
1,3-M_2-4-E-benzene	1054.2	1057.7	1059.6	1062.7

[a] M = methyl; E = ethyl; C_5 = pentane; C_6 = hexane; C_7 = heptane.

TABLE 8

RETENTION INDEX REPRODUCIBILITY (n = 5) AFTER WATTS AND SIMONICK[396]

Drugs were analysed under TPGC conditions using 5% phenyl- and 50% phenylmethylsilicone fused-silica capillary columns

Drug	*Retention index (i.u.)*			
	Ultra-2 (5% phenyl)		*HP-17 (50% phenyl)*	
	Mean	*S.D.*	*Mean*	*S.D.*
Amphetamine	368	0.55	436	0.55
Meperidine	615	0.55	712	0.00
Diazepam	878	0.00	1085	0.55
Cocaine	786	0.00	949	0.55
Phencyclidine	668	0.00	755	0.45
Morphine	873	0.00	1080	0.45
Lidocaine	663	0.55	776	0.89
Methaqualone	769	0.55	954	0.45
Methadone	764	0.00	882	0.45
Codeine	855	0.00	1048	0.45

stationary phases supplied commercially under the same trade name lead to unpredictable errors that are difficult to detect (Table 26).

After having studied the latter problem for several years, we have recently found[429b] a very simple and easy-to-use practical method for the characterization of stationary phases by introducing the so-called Kováts coefficient (eqn. 17a). In classical GLC, when the column temperature, inlet pressure and/or flow-rate of carrier gas are constant, the Kováts coefficient is constant. As can be deduced from eqn. 17a, the Kováts coefficient depends on the quality of both the stationary phase and the

TABLE 9

COMPARISON OF RETENTION INDICES MEASURED AND CALCULATED BY CHEN *ET AL.*[319] AND CALCULATED BY THE METHOD OF VAN DEN DOOL AND KRATZ[437] UNDER CONDITIONS OF TPGC ON CROSS-LINKED METHYLSILICONE

Compound	*Retention index (i.u.)*		
	Van den Dool and Kratz method	*Chen et al. method*	*Difference*
2-Methyl-2-butene	512.9	519.5	−6.6
2,3-Dimethylbutane	553.8	564.7	−0.9
2-Methylpentane	559.1	569.4	−10.3
3-Methylpentane	576.3	583.4	−7.1
2-Methyl-2-pentene	604.2	606.3	−2.1
2,2,5-Trimethylhexane	779.3	783.3	−4.0
n-Propylbenzene	933.3	935.6	−2.3
1-Methyl-4-ethylbenzene	944.8	946.3	−1.5

TABLE 10

COMPARISON OF RETENTION INDICES MEASURED AND CACULATED BY DONNELLY *ET AL.*[337] ON 5% PHENYLMETHYLSILICONE (DB-5) UNDER CONDITIONS OF TPGC

Compound[a]	*Retention index (i.u.)*		
	Measured	*Calculated*	*Difference*
2,7-PCDD	1985	1976	9
2,8-PCDD	1985	1976	9
1,2,4-PCDD	2152	2143	9
1,3,6,8-PCDD	2290	2290	0
1,3,7,9-PCDD	2304	2304	0
1,3,6,9-PCDD	2315	2319	−4
1,3,7,8-PCDD	2340	2338	2
1,2,7,9-PCDD	2364	2363	1
1,2,3,4,7-PCDD	2573	2576	−3

[a] PCDD = *p*-chlorodibenzodioxin.

carrier gas used, in addition to the column temperature. However, it is independent of all other GC parameters, including the type of *n*-alkanes applied for the determination of the *b* value (eqn. 17b), if $z \geqslant 8$. Similarly the Kováts coefficient is independent of the type of *n*-alkane applied for determination of the specific rention volume if $z \geqslant 8$. Some Kováts coefficients and retention polarities[429b] on various stationary phases are given in Table 27.

The dependence of the retention index on the column temperature can be described, in theory, by an Antoine-type equation[47]:

$$I = A + \frac{B}{T + C} \tag{1}$$

TABLE 11

COMPARISON OF RETENTION INDICES MEASURED AND CALCULATED BY WANG *ET AL.*[395] UNDER DIFFERENT TPGC CONDITIONS ON OV-101

Compound	*Retention index (i.u.)*		
	Constant inlet pressure	*Constant mass flow-rate*	*Difference*
Isobutyl acetate	754.5	755.6	−1.1
p-Xylene	867.0	867.8	−0.8
n-Heptanol	953.3	952.8	+0.5
Limonene	1030.9	1031.9	−1.0
Camphor	1134.9	1136.4	−1.5
Eugenol	1336.0	1336.7	−0.7
Diphenyl ether	1384.7	1385.2	−0.5
Caryophyllene	1435.2	1435.8	−0.6
β-Ionone	1472.8	1473.2	−0.4
n-Hexyl benzoate	1559.4	1559.9	−0.5

TABLE 12

COMPARISON OF RETENTION INDICES (i.u.) BY CHEN *ET AL.*[319] UNDER DIFFERENT TPGC CONDITIONS ON CROSS-LINKED METHYLSILICONE.

Initial temperature: 30°C.

Compound	*Heating rate (°C/min)*		
	2[a]	*5*[b]	*10*[a]
2-Methyl-2-butene	512.9	520.4	514.6
2-Methylpentane	559.1	569.5	561.5
3-Methylpentane	576.3	583.1	578.5
Benzene	639.3	648.0	645.6
Cyclohexane	647.7	656.4	655.3
cis-2-Heptene	706.3	712.1	709.4
Toluene	746.4	752.8	754.4
p-Xylene	852.0	854.3	860.6

[a] Calculation method of Van den Dool and Kratz (classical method)[437].
[b] Special calculation method of the authors.

where: I is the isothermal retention index (i.u.), T is the column temperature (K) and A, B and C are constants. Nevertheless, we emphasize again that in practical applications one is allowed to make use of the fact that on apolar stationary phases the Antoine equation can possess a wide linear range, within which the linear approximation can provide excellent results even without using the Antoine equation (Table 28).

TABLE 13

COMPARISON OF RETENTION INDICES MEASURED AND CALCULATED BY KRUPČIK *ET AL.*[358] UNDER TPGC CONDITIONS ON SQUALANE

Compound[a]	*Retention index (i.u.)*		
	Measured	*Calculated*	*Difference*
1,1,3-M_3-cyclo-C_5	722.2	721.8	0.4
1,*trans*-2,*cis*-4-M_3-cyclo-C_5	739.4	738.8	0.6
1,*trans*-2,*cis*-3-M_3-cyclo-C_5	746.1	746.7	−0.6
1,*cis*-2,*cis*-4-M_3-cyclo-C_5	775.6	777.7	−2.1
1,*cis*-2,*trans*-3-M_3-cyclo-C_5	780.0	781.1	−1.1
1,*cis*-3-M_2-cyclo-C_6	787.8	787.3	0.5
1,*trans*-4-M_2-cyclo-C_6	787.8	787.3	0.5
1,1-M_2-cyclo-C_6	790.6	792.4	−1.8
1-M-*trans*-3-E-cyclo-C_5	789.4	789.0	0.4
1-M-*cis*-3-E-cyclo-C_5	792.8	791.8	1.0
1-M-*trans*-2-E-cyclo-C_5	793.3	791.8	1.5
1-M-1-E-cyclo-C_5	797.2	796.9	0.3

[a] M = methyl; E = ethyl; C_5 = pentane; C_6 = hexane.

TABLE 14

COMPARISON OF RETENTION INDICES MEASURED AND CALCULATED BY BUCHMAN *ET AL.*[103] UNDER TPGC CONDITIONS ON SE-30 AND ON PEG-20M

Compound	*Retention index (i.u.)*			
	SE-30		*PEG-20M*	
	Measured	*Calculated*	*Measured*	*Calculated*
3-Iodotoluene	1148	1143	1674	1665
3-Bromotoluene	1033	1040	1498	1487
2-Cyclohexene-1-ol	880	890	1481	1499
3-Methylcyclohexanone	945	946	1376	1369
3-Methylcyclohexylamine	926	927	1231	1218
1,3-Dimethylcyclohexane	782	792	814	795
3-Methylcyclohexanol	947	950	1463	1450
1,3-Cyclohexanediol	1089	1108	2070	2105
4-Bromocyclohex-1-ene	969	974	1370	1388
3-Methyl-2-cyclohexen-1-one	1039	1026	1644	1587

TABLE 15

COMPARISON OF RETENTION INDICES MEASURED AND CALCULATED BY RAYMER *ET AL.*[213] ON SE-30 UNDER TPGC CONDITIONS.

Compound	*Retention index (i.u.)*		
	Measured	*Calculated*	*Difference*
2-Hexanone	600.0	588.3	11.7
4-Heptanone	681.5	686.6	−5.1
5-Nonanone	882.5	883.5	−1.0
6-Undecanone	1080.1	1080.4	−0.3
7-Tridecanone	1277.9	1277.2	0.7
8-Pentadecanone	1476.5	1474.1	2.4
9-Heptadecanone	1675.2	1671.0	4.2
10-Nonadecanone	1873.7	1867.8	5.9

TABLE 16

RETENTION INDICES OF SOME SUBSTITUTED PYRIDINES MEASURED BY PREMECZ AND FORD[372] UNDER TPGC CONDITIONS ON 5% PHENYLMETHYLSILICONE (DB-5) AND ON PEG-20M

Compound	*Retention index (i.u.)*	
	DB-5	*PEG-20M*
Pyridine	736.3	1182.7
2-Methylpyridine	813.5	1215.7
3-Methylpyridine	861.0	1283.5
4-Methylpyridine	861.9	1289.3
2,6-Dimethylpyridine	884.2	1243.1
2,4-Dimethylpyridine	931.8	1325.3
3,5-Dimethylpyridine	979.9	1390.8
3,4-Dimethylpyridine	1003.4	1444.9

TABLE 17

RETENTION INDICES OF SOME SULPHUR VESICANT AND RELATED COMPOUNDS ON DB-1 (100% DIMETHYLPOLYSILOXANE), DB-5 (95% METHYL-, 5% DIPHENYLPOLYSILOXANE) AND DB-1701 (86% DIMETHYL-, 14% CYANOPROPYLPHENYLPOLYSILOXANE), AFTER D'AGOSTINO AND PROVOST[398]

Column temperature, 2 min at 50°C, then programmed at 10°C/min to 300°C, maintained at 300°C for 5 min. Carrier gas, helium.

Compound	*Retention index (i.u.)*		
	DB-1	*DB-5*	*DB-1701*
1,4-Thioxane	850.7	880.0	964.1
2-Chloroethyl vinyl sulphide	873.7	903.0	992.1
(2-Vinylthio)ethanol	889.6	923.2	1089.2
Bis(2-chloroethyl) ether	948.3	984.0	1107.5
1,4-Dithiane	1018.7	1060.2	1156.7
1,4,5-Oxadithiapane	1117.8	1162.7	1289.9
Mustard	1123.8	1172.7	1325.8
Thiodiglycol	1130.9	1181.5	1458.2
Hemisulphur mustard	1132.3	1177.5	1400.0
1,2,5-Trithiapane	1300.0	1361.7	1513.3
Sesquimustard	1622.7	1688.8	1922.7

TABLE 18

RETENTION INDICES OF SOME AMINO ACID DERIVATIVES IN URINE MEASURED BY SCHNEIDER *ET AL.*[219] ON OV-101 UNDER TPGC CONDITIONS

Compound	*Retention index (i.u.)*	*Compound*	*Retention index (i.u.)*
Alanine	1159	Ornithine	1690
Glycine	1173	N(O)-TFA-tyrosine[a]	1769
α-Aminoisobutyric acid	1235	Glutamic acid	1807
Threonine	1246	Glutamine	1807
Serine	1257	Lysine	1811
Valine	1280	N-α-Methyllysine	1851
β-Aminoisobutyric acid	1282	2-Aminoadipic acid	1868
N-Methylproline	1328	1-Methylhistidine	1888
Leucine	1349	3-Methylhistidine	1888
Isoleucine	1358	Glycylproline	1931
Nicotinic acid	1384	N-TFA-Tyrosine[a]	1976
Creatinine	1440	Monoacylhistidine	1983
Aspartic acid	1676	N-α-Acetyllysine	2019
Asparagine	1676	Propylhydroxyproline	2058
Phenylalanine	1678		

[a] TFA = trifluoroacetyl.

TABLE 19

COMPARISON OF RETENTION INDICES (i.u.) MEASURED BY GNAUCK[109] WITH DIFFERENT HEATING RATES ON SE-30 UNDER TPGC CONDITIONS

Initial temperature, 363.16 K.

Compound[a]	*Heating rate (°C/min)*				*Standard deviation (i.u.) (n = 4)*
	4	*6*	*8*	*10*	
Allyl alcohol mono-EO-adduct acetate	981	980	981	983	2.26
Di-EO-adduct	1253	1253	1258	1252	2.41
3-EO-adduct	1526	1526	1522	1524	2.81
4-EO-adduct	1800	1799	1800	1800	2.30
5-EO-adduct	2080	2075	2075	2071	2.63

[a] EO = ethylene oxide.

TABLE 20

COMPARISON OF RETENTION INDICES (i.u.) MEASURED BY GNAUCK[109] WITH DIFFERENT INITIAL TEMPERATURES ON SE-30 UNDER TPGC CONDITIONS

Heating rate, 4°C/min.

Compound[a]	*Initial temperature (°C)*		
	50	*70*	*90*
Allyl alcohol mono-EO-adduct acetate	982	984	981
Di-EO-adduct	1258	1252	1253
3-EO-adduct	1524	1529	1526
4-EO-adduct	1803	1804	1800
5-EO-adduct	2080	2077	2080
6-EO-adduct	2349	2343	2344

[a] See Table 19.

TABLE 21

INFLUENCE OF THE SOLUTE SAMPLE SIZE ON RETENTION INDICES MEASURED BY WANG AND SUN[392] UNDER TPGC CONDITIONS ON OV-101

Compound	*Retention index (i.u.)*				
	OV-101				
	Sample size (ng)				
	0.1	*1.0*	*10*	*100*	*1500*
Isobutyl acetate	749.9	750.2	749.7	750.0	753.3
p-Xylene	859.6	859.9	859.8	860.5	864.7
Limonene	1026.2	1026.3	1026.3	1027.0	1031.8
Camphor	1129.5	1129.9	1129.7	1130.5	1136.4
n-Hexyl benzoate	1557.3	1557.2	1557.4	1559.8	1567.0

TABLE 22

RETENTION INDICES OF SOME MYCOTOXINS ON SE-52 DETERMINED BY BATA *ET AL.*[97] UNDER TPGC CONDITIONS

Toxin	*Retention index (i.u.)*	*Toxin*	*Retention index (i.u.)*
T-2 toxin	2822	Monoacetoxyscirpenol	2317
HT-2 toxin	2809	Scirpentriol	2260
Neosolaniol	2652	Fusarone-X	2485
T-2 tetraol toxin	2547	Deoxynivalenol	2323
Diacetoxyscirpenol	2421	Zearalenone	2867

TABLE 23

REPRODUCIBILITY OF RETENTION INDEX ON METHYLSILICONE (DB-1) UNDER TPGC CONDITIONS MEASURED BY NEWTON AND FOERY[134]

Compound	*Mean retention index (i.u.)*	*Standard deviation (i.u.)*	*Coefficient of variation (%)*	
			Within-run	*Day-to-day*
Barbital	1462.2	0.21	0.014	0.008
Butabarbital	1629.9	0.26	0.016	0.012
Pentobarbital	1711.2	0.27	0.016	0.010
Lidocaine	1853.0	0.28	0.015	0.004
Diazepam	2456.8	0.33	0.013	0.006

TABLE 24

COLUMN-TO-COLUMN REPRODUCIBILITY OF RETENTION INDEX ON 5% PHENYL-METHYLSILICONE (DB-5) UNDER TPGC CONDITIONS MEASURED BY NEWTON AND FOERY[134]

Compound	*Retention index (i.u.)*			
	Column 1	*Column 2*	*Column 3*	*Maximum range*
Barbital	1509.5	1511.6	1510.0	2.1
Lidocaine	1906.1	1905.4	1905.4	0.7
Propoxyphene	2232.9	2231.3	2229.3	3.6
Oxazepam	2386.9	2388.3	2387.0	1.4
Nordiazepam	2569.8	2566.3	2567.0	3.5

For example, the retention indices of 2,2,3-trimethylpentane on OV-101 at 40, 50 and 60°C are as follows:

$$I^{\text{OV-101}}_{2,2,3\text{-M3C5}}(40°\text{C}) = 733.4 \text{ i.u.} \quad (2)$$

$$I^{\text{OV-101}}_{2,2,3\text{-M3C5}}(50°\text{C}) = 734.3 \text{ i.u.} \quad (3)$$

$$I^{\text{OV-101}}_{2,2,3\text{-M3C5}}(60°\text{C}) = 735.1 \text{ i.u.} \quad (4)$$

TABLE 25

RETENTION INDEX SHIFTS AS A FUNCTION OF DRUG CONCENTRATION ON 5% PHENYLMETHYLSILICONE (DB-5) UNDER TPGC CONDITIONS MEASURED BY NEWTON AND FOERY[134]

Drug	*Retention index (i.u.)*		
	Concentration (ng/μl)		*Shift*
	5	*50*	
Ethchlorvynol	1024.0	1024.4	0.4
Nicotine	1342.7	1343.0	0.3
Barbital	1510.8	1514.5	3.7
Methylprylon	1553.8	1559.2	5.4
Propoxyphene	2225.1	2225.9	0.8
Lidocaine	1905.7	1904.7	−1.0
Morphine	2476.0	2481.3	5.3
Chlordiazepam	2575.3	2576.4	1.1

The Antoine equation of 2,2,3-trimethylpentane is

$$I^{\text{OV-101}}_{2,2,3\text{-M3C5}}(T) = 748.7 - \frac{2448.0}{T - 153.2} \tag{5}$$

The linear equation is

$$I^{\text{OV-101}}_{2,2,3\text{-M3C5}}(T) = -\frac{8870.8}{T} + 761.73 \tag{6}$$

The retention index differences were not significant. For example:

$$\text{at } 80°\text{C: } I(\text{Antoine}) - I(\text{linear}) = -0.1 \text{ i.u.} \tag{7}$$

$$\text{at } 100°\text{C: } I(\text{Antoine}) - I(\text{linear}) = -0.4 \text{ i.u.} \tag{8}$$

TABLE 26

INFLUENCE OF THE ORIGIN OF THE STATIONARY PHASE ON McREYNOLDS CONSTANTS DETERMINED ON DEGS AT 120°C, AFTER ETTRE[438]

Supplier	*McReynolds constant*				
	Benzene	*n-Butanol*	*2-Pentanone*	*Nitropropane*	*Pyridine*
Supelco (No. 1045)	1123	1295	1185	1440	1478
Chemical Research Services	1145	1323	1208	1485	1490
Supelco (No. 1303)	1149	1336	1217	1489	1534
PolyScience	1152	1341	1220	1492	1559

TABLE 27

KOVÁTS COEFFICIENTS ON SELECTED STATIONARY PHASES AT 120°C; FUNDAMENTAL DATA MEASURED BY McREYNOLDS[439]

Carrier gas, helium.

Stationary phase	*Kováts coefficient [dim]*	*Retention polarity (p.u.)*
Apiezon J	178.55	6.38
Apiezon L	166.87	4.39
Apiezon M	167.02	4.24
Apiezon N	175.95	6.67
Dioctyl sebacate	139.42	20.39
DC-550	193.94	19.25
Diisodecyl phthalate	194.59	23.93
Dioctyl phthalate	199.15	25.93
Tricresyl phosphate	262.54	44.34
UCON 50-HB-2000	253.93	49.39
NPGA	294.45	56.63
Pluronic-F88	288.60	58.70
Triton X-305	368.34	61.14
NPGS	336.72	65.23
PEG-20M	332.72	71.91
PEG-6000	330.20	71.95
XF-1150	370.55	75.43
PEG-1000	342.34	79.84
PEG-600	358.74	81.90
EGA	453.01	82.61
DEGA	455.52	85.61
PEG-1540	321.36	86.24
DEGS	614.53	106.63
PEG-4000	338.05	73.32

TABLE 28

COMPARISON OF MEASURED AND CALCULATED RETENTION INDICES OF 2,2,3-TRIMETHYLBUTANE ON APOLANE-87 STATIONARY PHASE AT DIFFERENT COLUMN TEMPERATURES

Column temperature (°C)	*Retention index (i.u.)*				
	Measured	*Calculated by linear equation*	*Difference*	*Calculated by Antoine equation*	*Difference*
50	639.2	638.9	0.3	639.2	0
70	642.1	642.3	−0.2	642.4	−0.3
90	645.5	645.6	−0.1	645.6	−0.1
110	648.9	649.0	−0.1	648.9	0
130	652.5	652.4	0.1	652.2	0.3
150	655.8	655.8	0	655.6	0.2
170	659.2	659.2	0	659.0	0.2

TABLE 29

RETENTION INDICES OF DIASTEREOMERS OF PRISTANE AND PHYTANE MEASURED BY BORWITZKY AND SHOMBURG[6] ON DIFFERENT STATIONARY PHASES AT DIFFERENT COLUMN TEMPERATURES

Stationary phase	*Retention index (i.u.)*							
	Pristane				*Phytane*			
	120°C	*130°C*	*150°C*	*180°C*	*120°C*	*130°C*	*150°C*	*180°C*
Apolane-87	–	–	1688.8	1689.2	–	–	1791.7	1792.6
	–	–	1690.2	1690.2	–	–	1793.3	1793.9
OV-101	1707.7	1708.4	–	–	1809.5	1810.6	–	–
	1709.4	1710.0	–	–	1811.6	1812.4	–	–

Sixty-seven *I versus* $1/T$ equations were presented by Rang *et al.*[373] for alkenes on OV-101 stationary phase.

Castello and Gerbino[404] studied the effect of column temperature on OV-1 and on SP-1000 stationary phase. An Antoine-type equation, the constants A, B and C of which can be calculated starting from experimental values, was found to be useful for the calculation of retention indices at any temperature on both polar and non-polar stationary phases. Their tables listed the constants of the Antoine equations which describe the dependence of the retention indices of 34 halogenated compounds on column temperature on OV-1 and SP-1000 stationary phases.

Ge *et al.*[108] used liquid crystal stationary phases to study the temperature dependence of the retention index. Chien *et al.*[54,55] studied the temperature dependence of the retention indices of selected compounds on OV stationary phases at different column temperatures.

Although many researchers have realized the necessity to carry out measurements at at least three different isothermal column temperatures for the given isothermal GC system being used[6,7,64,75,89,147,179,204,236,249,270,330,364], this prac-

TABLE 30

RETENTION INDICES OF SOME ALKYLNAPHTHALENES MEASURED BY BREDAEL[7] ON SE-30 AT DIFFERENT COLUMN TEMPERATURES

Compound	*Retention index (i.u.)*			
	80°C	*100°C*	*130°C*	*150°C*
2-Methylnaphthalene	1252	1265	1286	1300
1-Methylnaphthalene	1268	1281	1303	1318
2-Ethylnaphthalene	1328	1340	1358	1370
1-Ethylnaphthalene	1343	1357	1379	1393
2,3-Dimethylnaphthalene	1381	1395	1419	1433

TABLE 31

PRECISION OF RETENTION INDEX DETERMINATION OF TOLUENE MEASURED BY HAKEN *ET AL.*[64] ON A 10% SQUALANE COLUMN AT 100°C

Run No.	*Retention index (i.u.)*	*Run No.*	*Retention index (i.u.)*
1	760.3	7	760.6
2	760.4	8	760.7
3	760.5	9	760.6
4	760.4	10	760.6
5	760.4	11	760.4
6	760.4	12	760.6

Average 760.5
Standard deviation 0.14

TABLE 32

EFFECT OF VARYING THE SAMPLE TO *n*-ALKANE RATIO ON THE RETENTION INDEX OF *n*-HEPTANE MEASURED BY HAKEN *ET AL.*[64] ON SQUALANE AT 100°C

n-C_6:n-C_7:n-C_8 ratio	*Mean retention index (i.u.) (n = 10)*	*Standard deviation (i.u.)*
1:2:1	701.9	0.1
1:3:1	701.3	0.1
2:2:2	700.4	0.1

TABLE 33

COMPARISON OF RETENTION INDICES OF SOME AROMATICS MEASURED BY LUBECK AND SUTTON[75] ON 5% PHENYLMETHYLSILICONE (DB-5) WITH DIFFERENT FILM THICKNESSES AT 60°C

Compound	*Retention index (i.u.)*		
	Column 1 (0.25 μm)	*Column 2 (1.00 μm)*	*Difference*
Benzene	670.6	670.4	0.2
Toluene	772.7	772.8	−0.1
Ethylbenzene	865.0	865.1	−0.1
m-Xylene	872.4	872.4	0
p-Xylene	873.0	873.0	0
o-Xylene	895.7	895.8	−0.1
1,3,5-Trimethylbenzene	968.8	968.8	0
1,2,4-Trimethylbenzene	992.6	992.7	−0.1

TABLE 34

RETENTION INDICES OF *n*-ALKYLBENZENES MEASURED BY VERNON AND SURATMAN[89] ON APIEZON-L AND ON PEG-20M AT DIFFERENT COLUMN TEMPERATURES

Compound	*Retention index (i.u.)*					
	110°C		*130°C*		*150°C*	
	APL	*PEG-20M*	*APL*	*PEG-20M*	*APL*	*PEG-20M*
Benzene	687.2	974.2	693.7	983.4	700.2	992.6
Toluene	795.6	1073.3	802.3	1083.4	809.0	1093.5
Ethylbenzene	885.4	1158.9	892.6	1169.8	899.8	1180.7
n-Propylbenzene	–	–	981.5	1250.7	988.8	1261.6

tical solution has not found general application, mostly owing to the time shortage involved. Some interesting results are summarized in Tables 29–43. See also refs. 147, 266, 275, 276, 321, 353, 404 and 462.

5. ERRORS IN RETENTION INDEX DETERMINATION

As in our previous review[47], this is still one of the most frequently discussed aspects of retention index systems. In the last 5 years many researchers have dealt with the accuracy and reproducibility of retention index determinations and the following

TABLE 35

EFFECT OF SAMPLE PROPORTIONS MEASURED BY VERNON AND SURATMAN[89] ON SQUALANE AT 100°C

Components	*Proportions*	*Retention index (i.u.)*	*Standard deviation (i.u.) (n = 12)*
C_7:benzene:C_8	1:1:1	706.1	0.2
	1:1:2	705.2	0.4
	1:1:3	704.5	0.5
	1:2:1	704.2	0.4
	1:3:1	703.0	0.6
	2:2:1	707.1	0.4
	2:1:1	709.4	0.2
	3:1:1	711.1	0.1
C_7:1-propanol:C_8	1:1:1	694.8	0.1
	1:2:1	694.4	0.2
	2:2:1	696.9	0.4
	3:1:1	697.6	0.2
C_7:2-pentanone:C_8	1:1:1	768.3	0.2
	1:1:3	764.1	0.4
	2:1:1	768.1	0.5

TABLE 36

RETENTION INDICES OF SOME MYCOTOXINS ON SE-52 STATIONARY PHASE AT DIFFERENT COLUMN TEMPERATURES MEASURED BY TAKÁCS *ET AL.*[147]

Toxin[a]	*Retention index (i.u.)*			
	190°C	*210°C*	*230°C*	*250°C*
Deoxynivalenol-TMS	2317.3	2330.0	2346.2	2367.8
Diacetoxycirpenol-TMS	2421.9	2430.0	2437.1	2444.0
Fusarenone-X-TMS	2439.5	2456.9	2475.0	2493.9
HT-2-TMS	2800.3	2817.5	2836.4	2858.3
T-2-TMS	2803.6	2815.9	2832.0	2853.8

[a] TMS = Trimethylsilyl derivative.

TABLE 37

RETENTION INDICES OF SATURATED C_3–C_5 BRANCHED-CHAIN ALCOHOLS ON SE-30 AT DIFFERENT COLUMN TEMPERATURES, AFTER HAKEN AND KORHONEN[179]

Alcohol	*Retention index (i.u.)*					
	60°C	*80°C*	*100°C*	*120°C*	*140°C*	*160°C*
2-Propanol	491	453	508			
2-Methyl-2-propanol	531	500	548			
2-Butanol	605	577	615	622		
2-Methyl-2-butanol	644	628	652	662	642	
2-Methyl-1-propanol	629	609	629	641	620	
3-Methyl-2-butanol	683	668	683	690	699	
2-Pentanol	697	683	692	701	704	
3-Methyl-1-butanol	733	721	723	727	738	

TABLE 38

PRECISION OF RETENTION INDEX DETERMINATION AS THE STANDARD DEVIATION (n = 5) ON OV-101 AND OV-17 STATIONARY PHASES AT 200°C MEASURED BY MACEK AND SMOLKOVÁ-KEULEMANSOVÁ[204]

Compound	*Retention index (i.u.)*		*Standard deviation (i.u.)*	
	OV-101	*OV-17*	*OV-101*	*OV-17*
2-Phenylpropionic acid	1299.7	1453.0	±1.2	±0.9
2-(4-Methylphenyl)propionic acid	1385.6	1547.8	±0.7	±0.9
2-(4-Ethylphenyl)propionic acid	1473.1	1634.2	±0.8	±0.4
2-(4-Isobutylphenyl)propionic acid	1614.3	1751.9	±0.3	±0.8
2-(4-Methoxyphenyl)propionic acid	1528.6	1735.2	±0.2	±0.3

TABLE 39

RETENTION INDICES OF SOME α-HALOGENOCARBOXYLIC ACIDS MEASURED BY WATABE AND GIL-AV[236] ON (*R*)-N-LAUROYL-α-(1-NAPHTHYL)ETHYLAMINE AT DIFFERENT COLUMN TEMPERATURES

tert.-Butylamide	*Retention index (i.u.)*		
	110°C	*130°C*	*150°C*
(*S*)-α-Chloropropanoic acid	1264	1263	1268
(*R*)-α-Chloropropanoic acid	1275	1271	1277
(*S*)-α-Chloro-*n*-butanoic acid	1353	1353	1355
(*R*)-α-Chloro-*n*-butanoic acid	1365	1363	1364
(*S*)-α-Chloroisopentanoic acid	1394	1399	1403
(*R*)-α-Chloroisopentanoic acid	1405	1409	1411
(*S*)-α-Chloro-*n*-pentanoic acid	1441	1444	1444
(*R*)-α-Chloro-*n*-pentanoic acid	1451	1453	1454

TABLE 40

RETENTION INDICES OF SOME ALKENES ON OV-101 AND SQUALANE STATIONARY PHASES AT 50 AND 70°C, AFTER BONEVA AND DIMOV[249]

Compound	*Retention index (i.u.)*			
	OV-101		*Squalane*	
	50°C	*70°C*	*50°C*	*70°C*
trans-2-Pentene	507.0	506.8	500.2	500.0
cis-2-Pentene	512.0	512.4	505.0	505.2
4-Methyl-*cis*-2-pentene	565.6	566.1	556.2	556.6
4-Methyl-*trans*-2-pentene	569.1	568.8	562.1	561.9
cis-3-Hexene	602.4	602.7	592.8	593.1
trans-3-Hexene	602.8	602.4	592.5	591.9
cis-2-Hexene	613.6	614.3	604.0	614.6
3-Methyl-*trans*-2-pentene	620.6	620.9	613.1	613.3
4,4-Dimethyl-*trans*-2-pentene	621.3	621.2	614.7	614.7
4,4-Dimethyl-*cis*-2-pentene	645.1	646.9	635.8	637.8
4-Methyl-*trans*-2-hexene	665.0	665.6	657.0	657.6
4-Methyl-*cis*-2-hexene	660.0	661.2	655.1	656.1
3,4-Dimethyl-*trans*-2-pentene	685.1	685.8	678.0	678.7
3,4-Dimethyl-*cis*-2-pentene	676.7	677.7	670.9	671.7
3-Methyl-*cis*-3-hexene	691.0	691.3	684.8	685.1
3-Methyl-*cis*-2-hexene	701.0	701.7	693.8	694.4
2,2-Dimethyl-*cis*-3-hexene	723.2	724.7	717.0	718.7
2,5-Dimethyl-*trans*-3-hexene	703.7	703.2	695.0	694.5
trans-2-Heptene	709.0	709.2	698.6	698.7
cis-3-Heptene	696.1	696.9	690.5	691.1
trans-3-Heptene	694.0	694.2	687.5	687.5
trans-4-Octene	792.8	793.1	783.9	784.1

can be mentioned: Huizing[18], Bogusz *et al.*[42a], Enqvist *et al.*[56], Hangac *et al.*[66], Knoeppel *et al.*[70], Vernon and Suratman[89], Svetlova *et al.*[145], Bermejo and Guillen[165], Asselin[244], Smith *et al.*[308] and Wang and Sun[392].

Additional interesting data were published by Larson *et al.*[474], Enqvist and

TABLE 41

RETENTION INDICES OF SOME C_1–C_8 MONOCHLORINATED *n*-ALKYL ACETATES ON SE-30 AND OV-351 AT DIFFERENT COLUMN TEMPERATURES, AFTER HAKEN AND KORHONEN[270]

Isomeric acetate	*Retention index (i.u.)*							
	SE-30			*OV-351*				
	60°C	*80°C*	*100°C*	*60°C*	*80°C*	*100°C*		
Methyl	508	505	505	823	839	844		
Chloro	691	680	674	1181	1180	1164		
Ethyl	613	577	607	881	880	875		
1-Chloro	744	724	726	1150	1153	1123		
2-Chloro	817	801	796	1315	1319	1297		
Propyl	711	685	676	981	978	943		
1-Chloro	838	821	806	1217	1222	1198		
2-Chloro	864	848	835	1296	1304	1283		
3-Chloro	924	916	902	1418	1425	1414		
	SE-30				*OV-351*			
	80°C	*100°C*	*120°C*	*140°C*	*80°C*	*100°C*	*120°C*	*140°C*
Butyl	810	786	774	781	1079	1053	1064	1154
1-Chloro	930	906	895	912	1298	1278	1279	1342
2-Chloro	964	944	930	951	1384	1370	1375	1425
3-Chloro	981	961	949	972	1432	1422	1427	1478
4-Chloro	1038	1021	1008	1033	1539	1533	1540	1585
Pentyl	912	885	867	879	–	1195	1182	1212
1-Chloro	1026	1006	990	1011	–	1401	1387	1419
2-Chloro	1051	1033	1017	1041	–	1469	1460	1492
3-Chloro	1078	1062	1048	1072	–	1528	1525	1558
4-Chloro	1089	1073	1058	1084	–	1563	1564	1594
5-Chloro	1143	1129	1116	1142	–	1652	1657	1687
	SE-30				*OV-351*			
	100°C	*120°C*	*140°C*	*160°C*	*100°C*	*120°C*	*140°C*	*160°C*
Hexyl	1008	972	993	1000	1277	1255	1286	1295
1-Chloro	1117	1086	1108	1114	1472	1457	1492	1500
2-Chloro	1147	1121	1143	1151	1540	1532	1576	1574
3-Chloro	1166	1143	1166	1173	1581	1578	1627	1629
4-Chloro	1186	1163	1187	1194	1626	1627	1679	1679
5-Chloro	1194	1171	1194	1202	1647	1650	1700	1703
6-Chloro	1247	1227	1249	1257	1747	1737	1789	1796

(*Continued on p. 24*)

TABLE 41 (*continued*)

Isomeric acetate	*Retention index (i.u.)*									
	SE-30					*OV-351*				
	100°C	*120°C*	*140°C*	*160°C*	*180°C*	*100°C*	*120°C*	*140°C*	*160°C*	*180°C*
Heptyl	1095	1070	1086	1090	1093	1385	1372	1401	1400	1385
1-Chloro	1211	1191	1211	1215	1212	1582	1578	1602	1607	1615
2-Chloro	1235	1217	1239	1244	1242	1647	1647	1673	1673	1675
3-Chloro	1254	1238	1260	1266	1262	1686	1691	1718	1720	1725
4-Chloro	1266	1251	1273	1278	1276	1717	1723	1753	1757	1759
5-Chloro	1284	1269	1292	1297	1295	1749	1756	1787	1791	1797
6-Chloro	1291	1276	1298	1303	1299	1767	1775	1806	1809	1818
7-Chloro	1342	1329	1350	1354	1354	1846	1857	1889	1894	1904
Octyl	1194	1165	1188	1189	1190	1468	1454	1490	1486	1485
1-Chloro	1306	1287	1312	1314	1315	1659	1658	1702	1704	1711
2-Chloro	1328	1312	1338	1343	1344	1722	1724	1771	1771	1775
3-Chloro	1345	1331	1358	1362	1364	1758	1764	1814	1819	1824
4-Chloro	1355	1342	1369	1373	1373	1787	1794	1845	1850	1856
5-Chloro	1365	1352	1379	1384	1385	1802	1811	1862	1867	1874
6-Chloro	1382	1370	1396	1402	1405	1830	1840	1893	1899	1909
7-Chloro	1385	1372	1399	1403	1405	1842	1852	1905	1912	1922
8-Chloro	1435	1452	1451	1456	1458	1918	1932	1987	1994	2006

TABLE 42

RETENTION INDICES OF C_4–C_5 EPOXIDES ON OV-101 STATIONARY PHASE AT DIFFERENT COLUMN TEMPERATURES MEASURED BY BONEVA AND DIMOV[330]

Compound	*Retention index (i.u.)*		
	70°C	*80°C*	*90°C*
2-Methyl-1,2-epoxypropane	534.4	534.7	535.0
trans-2,3-Epoxybutane	543.5	543.6	543.7
1,2-Epoxybutane	587.0	587.4	587.8
cis-2,3-Epoxybutane	572.6	573.1	573.6
3-Methyl-1,2-epoxybutane	612.0	613.3	613.8
1,2-Epoxypentane	624.5	624.7	624.9
2-Methyl-1,2-epoxybutane	633.3	633.7	634.0
trans-2,3-Epoxypentane	654.9	655.0	655.1
cis-2,3-Epoxypentane	658.4	659.4	660.4
2-Methyl-2,3-epoxybutane	668.0	668.1	668.2

TABLE 43

RETENTION INDICES OF SOME DRUGS ON SE-30 AT DIFFERENT COLUMN TEMPERATURES MEASURED BY MUSUMARRA *ET AL.*[364]

Drug	*Retention index (i.u.)*		
	140°C	*160°C*	*180°C*
Amantadine	1272	–	–
Amphetamine	1115	–	–
Beclamide	–	–	1697
Caffeine	–	–	1809
Clobutinol	–	–	1758
Cropropamide	–	–	1706
Crotethamide	–	–	1661
Cyclopentamine	1100	–	–
Ephedrine	1388	–	–
Ethosuximide	1250	–	–
Fenfluramine	–	1240	–
Heptaminol	1188	–	–
Hordenine	–	1495	–
Ketamine	–	–	1820
Lefetamine	–	1670	–
Lidocaine	–	–	1875
Meperidine	–	–	1741
Mephentermine	–	1305	–
Methamphetamine	1188	–	–
Methoxyphenamine	–	1390	–
Metronidazole	–	–	1618
Mexiletine	1408	–	–
Nicametate	–	1648	–
Nicotine	–	1360	–
Nikethamide	1520	–	–
Oxyfedrine	1380	–	–
Phenacetin	–	–	1695
Phendimetrazine	–	1450	–

Hesso[12], Enqvist *et al.*[174], Haken *et al.*[64], Hangac *et al.*[66], Pacholec and Poole[135], Ballschmiter *et al.*[161] and Dimov[261].

6. DETERMINATION AND/OR CALCULATION OF THE GAS HOLDUP (DEAD) TIME

Determination and/or calculation of the gas holdup (dead) time (t_M) is still a particularly widely discussed problem[149,150,222,224,230,312], as reported in the previous review also[47]. Computer-assisted studies are of special importance.

Tóth and Zala[149,150] dealt with the calculation of the constants of the *n*-alkane retention time curve and the gas holdup time in GLC. The numerical results obtained with their calculation method were compared with those obtained by Gröbler and Bálizs' iterative method[440] and the results agreed well.

Smith *et al.*[222] compared some mathematical methods for the calculation of dead time and Kováts retention indices[224]. They also dealt with the linearity of the plot

of log(adjusted retention time) *versus* carbon number for *n*-alkanes. Seven methods, together with two modified approaches allowing the optimization of t_M while simultaneously fitting a cubic or fifth-degree polynomial to the data, were compared[222]. Their studies and results are of great interest and their review is the most important paper in this field.

Summarizing the results on gas holdup times, it can be stated that if correct retention time data are available, the simple mathematical method applying three consecutive *n*-alkanes is equivalent to the more complicated computer methods. With incorrect retention time data, even a skilful computer operator is unable to produce correct retention index values. Therefore, it is essential to select correct, calculated or determined gas holdup times for the calculations of exact retention indices.

7. SLOPE OF THE *n*-ALKANE PLOT. THE *b* VALUE. TEMPERATURE DEPENDENCE OF THE *b* VALUE ON DIFFERENT STATIONARY PHASES

By the *n*-alkane plot we mean the plot of $\log X(z)$ *versus* carbon number (z) relationship for *n*-alkanes, where X is the retention value used in the calculation. In the most frequent treatment, values of the adjusted retention times (t'_R) or net retention times (t_N), and also values of specific (V_g) or net retention volumes (V_N) should be substituted for X.

The *n*-alkane plots are generally linear except for the first seven members of the homologous series[47]. The reason is that for the *n*-alkanes of low carbon number, owing to the individual bonds in their molecules, the deviation from linearity decreases from methane to *n*-hexane. Previously, we described the structure of the first eight *n*-alkanes[451] and their bond codes[385]. It can be seen that the first six members of the homologous series (up to *n*-hexane) contain different types of individual bonds. Thus, they will interact differently with the stationary phase, and therefore, the relationship cannot be linear. Linear relationships start with *n*-heptane; from here on, each *n*-alkane molecule contains the same types of bonds and they differ from each other only in the number of these bonds.

The dependence of the *b* value on the column temperature can be described, in theory, by an Antoine-type equation, similarly to the retention index and Kováts coefficient[47]. Nevertheless, the temperature dependence of the *b* value can be approximately described with the following equation:

$$b_T^{\text{st.ph.}} = \frac{D}{T} + E \tag{9}$$

where D and E are constants and T is the column temperature (K). Eqn. 9 is a practical possibility for checking the effective column temperature, as

$$T = \frac{D}{b_T^{\text{st.ph.}} - E} \tag{10}$$

The equations used for temperature control of columns with some OV stationary phases are as follows:

$$\text{OV-1: } T = \frac{215.97}{b + 0.2898} \tag{11}$$

$$\text{OV-3: } T = \frac{238.40}{b + 0.3452} \tag{12}$$

$$\text{OV-7: } T = \frac{225.62}{b + 0.3037} \tag{13}$$

$$\text{OV-11: } T = \frac{234.12}{b + 0.3293} \tag{14}$$

$$\text{OV-22: } T = \frac{245.44}{b + 0.3769} \tag{15}$$

$$\text{OV-25: } T = \frac{277.26}{b + 0.4732} \tag{16}$$

We compared the calculated data obtained by using different equations and the results are given in Table 44.

Morishita *et al.*[78] achieved good results in this field for chlorinated alkanes on PEG-20M and Apiezon L stationary phases at 90°C. Berezkin and Retunsky[163] checked the linearity of the *n*-alkane plot on four different stationary phases and the temperature dependence of the *b* value, using *n*-alkanes, 1-alkanols and their acetates as markers. The linearity of the *n*-alkane plot and the problem of the *b* value were studied on various stationary phases at different column temperatures by Hawkes[413], who obtained satisfactory results (Table 45).

Touabet *et al.*[430] studied methods for testing the linearity of retention time *versus* carbon number plots for *n*-alkanes. Through statistical evaluation of the results, four parameters models were generally found to be adequate. Engewald *et al.*[340] compared the *b* values with ΔG_{CH_2} values on different stationary phases at 100°C

TABLE 44

COMPARISON OF *b* VALUES MEASURED BY CHIEN *ET AL.*[54,55] AND CALCULATED BY VARIOUS METHODS ON OV-25 STATIONARY PHASE AT DIFFERENT COLUMN TEMPERATURES

Column temperature (°C)	*b Value*				
	Measured	*Calc. by linear equation*	*Difference*	*Calc. by Antoine equation*	*Difference*
40	0.4120	0.4122	−0.0002	0.4121	−0.0001
60	0.3590	0.3590	0	0.3590	0
80	0.3130	0.3119	0.0011	0.3130	0
100	0.2710	0.2698	0.0012	0.2728	−0.0018
120	0.2350	0.2320	0.0030	0.2374	−0.0024

TABLE 45

b VALUES FOR VARIOUS STATIONARY PHASES AT DIFFERENT COLUMN TEMPERATURES, AFTER HAWKES[413]

Stationary phase	*b Value*					
	50°C	*75°C*	*80°C*	*100°C*	*120°C*	*125°C*
Squalane	0.436	0.386	0.361	0.331	0.289	–
Apiezon L	0.432	0.370	–	0.315	–	0.263
OV-101	0.369	0.317	–	0.275	–	0.226
OV-3	0.388	0.323	–	0.277	–	0.239
OV-7	0.396	0.325	–	0.276	–	0.241
OV-61	0.340	0.272	–	0.239	–	0.207
OV-11	0.343	0.318	–	0.279	–	0.236
OV-17	0.379	0.310	0.318	0.276	0.261	0.228
OV-22	0.343	0.276	–	0.240	–	0.211
OV-25	0.306	0.246	–	0.227	–	0.188
OV-210	0.297	0.227	–	0.202	–	0.171
OV-225	0.339	0.253	–	0.226	–	0.186
Silar-5CP	0.321	0.276	–	0.216	–	0.180
Silar-10CP	0.261	0.221	–	0.177	–	0.137
PEG-20M	–	0.259	–	0.225	0.193	0.190
QF-1	–	0.289	0.262	–	0.209	–
DEGS	–	–	–	–	0.193	–

(Table 46). The *b* values measured by Chien *et al.*[54,55] and Hawkes[413] are summarized in Table 47. Finally, some *b* values measured by Chien *et al.*[54,55] on different OV stationary phases at different column temperatures are given in Table 48.

8. CONNECTION BETWEEN PHYSICO-CHEMICAL QUANTITIES AND RETENTION INDEX

In theory, this is one of the most important questions with respect to retention index system. A fundamental relationship exists between the specific retention volume (V_g) and the retention index (I):

$$\log V_g(s) = \frac{(I - K_c)b}{100} \tag{17}$$

TABLE 46

COMPARISON OF *b* VALUES AND ΔG_{CH_2} VALUES ON DIFFERENT STATIONARY PHASES AT 100°C, AFTER ENGEWALD *ET AL.*[340]

Stationary phase	*b Value*	ΔG_{CH_2} *(J mol^{-1})*
Squalane	0.3196	2280
UCON LB-550X	0.2916	2080
UCON 50-HB-280X	0.2804	2000
OV-1	0.2776	1980
PEG-20M	0.2467	1760
Carbopack C (GTCB)	0.5860	4180

TABLE 47

COMPARISON OF *b* VALUES MEASURED BY CHIEN *ET AL.*[54,55] AND HAWKES[413] ON DIFFERENT OV STATIONARY PHASES AT 50°C

Stationary phase	*b Value*		
	Chien et al.[54,55]	*Hawkes*[413]	*Difference*
OV-101	0.3847	0.369	0.0157
OV-3	0.3924	0.388	0.0044
OV-7	0.3947	0.396	−0.0013
OV-11	0.3951	0.343	0.0521
OV-17	0.3886	0.379	0.0096
OV-22	0.3826	0.343	0.0396
OV-25	0.3846	0.306	0.0786

where K_c is the Kováts coefficient[429b]:

$$K_c = 100\left[z - \frac{\log V_g(z)}{b}\right] \tag{17a}$$

and b is the slope of the linear section of the *n*-alkane plot:

$$b = \log V_g(z + 1) - \log V_g(z) \tag{17b}$$

A useful relationship is available, if the following data for the GLC system used are known:

$$I_{s1}^{st.ph.}(T) - I_{s2}^{st.ph.}(T) = \frac{100}{b} \cdot \log \alpha \tag{18}$$

TABLE 48

b VALUES FROM DATA MEASURED BY CHIEN *ET AL.*[54,55] ON DIFFERENT STATIONARY PHASES AT DIFFERENT COLUMN TEMPERATURES

Stationary phase	*Phenyl (%)*	*b Value*			
		30°C	*50°C*	*70°C*	*90°C*[a]
OV-101	0	0.4323	0.3847	0.3427	0.3055
OV-1	0	0.4226	0.3785	0.3395	0.3048
OV-3	10	0.4415	0.3924	0.3498	0.3124
OV-7	20	0.4396	0.3947	0.3530	0.3141
OV-11	35	0.4430	0.3951	0.3530	0.3157
OV-17	50	0.4311	0.3886	0.3478	0.3086
OV-22	65	0.4327	0.3826	0.3383	0.2988
OV-25	75	0.4419	0.3846	0.3352	0.2922

[a] Values calculated by Antoine equations from measured data.

where α is relative volatility. In this rather broad area of research, numerous papers have been devoted clarifying the relationship between the boiling point and the retention index. Without aiming at completeness, we cite the contributions of Bermejo and Guillen[42], Calixto *et al.*[51], Chelghoum *et al.*[258], Nabivach and Vasiliev[365].

A method for calculating "solubility factors" in GLC by using retention indices was developed by Patte *et al.*[28a]. This method was refined and applied to 240 selected compounds covering a wide range of functional groups and molecular structures. Retention indices of mono- and bifunctional molecules on SE-30, squalane, PEG-20M and DEGS at 120°C were related to structural parameters by means of multiple regression and factor analysis by Buydens and co-workers[49,50].

Golovnya and Grigoryeva[58] derived a universal equation for members of homologous series of some organic compounds permitting the calculation of specific retention volume (V_g), retention index (I), partial free energy of sorption and the change in the energy contribution of a methylene unit. A significant correlation was found between the retention indices and the average molecular polarizabilities of ten polycyclic aromatic hydrocarbons by Lamparczyk *et al.*[72] on SE-30, OV-101, SE-52, OV-7 and OV-17 at 250°C. Szász *et al.*[84] demonstrated a relationship between molecular connectivity index, partition coefficient and chromatographic parameters. Berezkin and Retunsky[99] derived a modified equation for the calculation of the theoretical plate number and selection of the sorbent on the basis of retention indices.

Bermejo *et al.*[101] studied the relationships between parameters related to electronic polarizability, such as molar refraction, refractive index, Van der Waal's volume and molar volume, and the retention indices of alkylbenzenes on squalane and PEG-20M at 100°C. Good correlations were found by Calixto *et al.*[104] between retention indices and the total and binding energies. An interesting paper was published by Lamparczyk and Radecki[126] on correlations between retention and molecular physico-chemical properties such as polarizability and dipole moment.

Molecular orbital calculations were used to study the retention of *n*-alkenes by Garcia-Raso *et al.*[138]. Using the retention index, the influence of molecular parameters such as total energy, binding energy, excess charge distribution and values of the energy and coefficients of the highest occupied and lowest unoccupied molecular orbitals were considered. According to Klopman's equation, these parameters are directly related to the solute–stationary phase interaction term of the retention index. General equations including several homologous series were presented.

Sabljić[141] studied the calculation of the retention indices of chlorinated alkanes by molecular topology. The comparison of the results obtained with the molecular connectivity model and the empirical additive scheme revealed important advantages of the molecular connectivity approach[214].

Buydens *et al.*[169] presented a relationship between GC behaviour and topological, physico-chemical and quantum chemically calculated charge parameters for neuroleptica. Retention indices of some neuroleptic drugs were subsequently measured on OV-101 and OV-17 at 260°C[255].

Based on the influence of the binding energy on retention, relationships between the retention indices on squalane and the heats of formation of some hydrocarbons were found by Calixto *et al.*[170a]. Terms taken from the information theory[456] were used in order to improve the correlation coefficients of the equations of retention index *versus* heat of formation.

For a structurally diverse set of primary, secondary and tertiary heterocyclic amines, correlations were found by Osmialowski *et al.*[209] between retention indices and quantum chemically calculated parameters. Golovnya[267a] suggested a universal equation describing the deviation from linearity of the plots for *n*-alkanes and other homologous series. She demonstrated the principle of non-additivity of the sorption energy and its application to the precalculation of retention indices. Retention indices for seven straight-chain liquid-phase solvents (*n*-heptadecane, 1-hexadecene, 1-hexadecyl chloride, 1-hexadecyl bromide, 1-hexadecyl iodide, di-*n*-octyl ether and di-*n*-octyl thioether) of comparable length and 49 straight- and branched-chain solutes were target factor analysed by Howery and Soroka[279]. (Target factor analysis is a mathematical computer method for analysis of experimental errors. It was introduced in the research of retention indices, McReynolds system, etc. by Malinowski and Howery[490]; *cf.* refs. 47 and 567–570).

Robbat *et al.*[301] derived a multivariate relationship between the retention indices and molecular connectivities of mononitrated polycyclic aromatic hydrocarbons. Bermejo and Guillen[326] obtained valuable results by applying the regular solution model to the solution processes that take place in GLC. Equations were obtained that relate the specific retention volume of solutes to their molecular volume and latent heat of vaporization. Papp and co-workers[367,368] studied the correlations between molar refraction and retention indices measured on OV-1 and OV-225 at 240°C. They reported[368] some problems in the correlation of molecular parameters and the connectivity index. Sanchez *et al.*[378] studied the effect of the number of McReynolds constants on the reproducibility on 250 stationary phases.

Model non-ionic surfactants were analysed by Voelkel[387] on four stationary phases (SE-30, Apiezon K, OV-17 and QF-1) at different column temperatures. The retention indices and the partial molar thermodynamic parameters of solution, *i.e.*, Gibbs free energy, enthalpy and entropy, and their increments for characteristic structural fragments were calculated.

For further details, see refs. 22, 31, 35, 37, 69, 73, 98, 107, 139, 164, 212, 212a, 231, 242, 287, 294, 317, 328, 359, 359a, 360, 373, 390 and 397.

9. RETENTION INDEX AND MOLECULAR STRUCTURE

The relationship between molecular structure and retention index is the most frequently discussed aspect of retention index systems and remains at the centre of interest. Research activity in this area can be classified according to the basic idea from which the calculations and experimental studies started.

In the simplest case, the basis of research is the various differences between the values of the retention index of the many studies of this type we refer to those made by Boneva, Dimov and co-workers[44,249,260,329], Engewald and co-workers[339,340,409] and Haken, Korhonen and co-workers[60–63,111–113,119–125,177–179,180,185–196,282–284].

Useful retention indices published by Supelco[225a] are given in Table 49.

Retention indices of alkylbenzenes measured on various stationary phases at different column temperatures are of continuous interest for researchers and research teams. Remarkable results are shown in Tables 50–57.

Structure–retention correlations were derived from retention index differences

TABLE 49

RETENTION INDICES OF VARIOUS COMPOUNDS ON SP-1700 at 70°C[225a]

Compound	*Retention index (i.u.)*	*Compound*	*Retention index (i.u.)*
Ethylene	270	1,3-Butadiene	479
Carbon dioxide	281	Pentene-1	517
Propylene	329	*trans*-2-Pentene	530
Acetylene	336	2-Methyl-1-butene	530
Isobutane	366	*cis*-2-Pentene	546
Carbonyl sulphide	380	2-Methyl-2-butene	558
Isobutene/1-butene	420	2-Methylpentane	566
trans-2-Butene	443	3-Methylpentane	585
cis-2-Butene	460	3-Methylhexane	655
Isopentane	470		

by Engewald *et al.*[340]. It was shown that the combined application of retention index differences obtained in GLC and gas–solid chromatography (GSC) provides more detailed structural information (Table 58).

The GC retention behaviour of a series of chlorinated phenyl acetates was studied by Haken and Korhonen[61] on SE-30 and OV-351 at different column temperatures (Table 59). Haken and Korhonen[62] also dealt with the determination of isomeric chlorobenzenes on SE-30 and PEG-20M at different column temperatures and some of the retention indices measured are presented in Table 60. Retention indices of some aromatic esters measured by Haken *et al.*[65] on SE-30, OV-25, QF-1 and Silar 10C stationary phases at different column temperatures, *e.g.*, 200°C, are shown in Table 61. Retention indices of perfluoro-*n*-alkanes measured by Müller *et al.*[79] are listed in Table 62.

By means of GLC with a combination of SE-30 with three different stationary phases (Wax-51, Siponate DS-10 and OV-215), various compounds and chemical classes were identified from differences in retention indices by Winskowski[93]. Some of

TABLE 50

RETENTION INDICES OF SOME AROMATICS MEASURED BY BREDAEL[7] ON SE-30 AT 80 AND 130°C

Compound	*Retention index (i.u.)*	
	80°C	*130°C*
Benzene	659	670
Toluene	761	774
Ethylbenzene	854	867
m-Xylene	864	876
p-Xylene	864	876
o-Xylene	885	900
1,3,5-Trimethylbenzene	960	972
1,2,4-Trimethylbenzene	984	1000
1,2,3-Trimethylbenzene	1009	1029

TABLE 51

RETENTION INDICES (i.u.) OF SOME GEOMETRIC ISOMERS IN THE NEMATIC RANGE OF SOME SPECIAL STATIONARY PHASES AND ON OV-225 AT 99°C, AFTER ISENBERG *ET AL.*[117]

Substance	*Stationary phase*		
	4-n-Dodecyl-benzoic acid	*2-Chlorohydro-quinonebis(4-n-octyloxy benzoate)*	*OV-225*
m-Xylene	932	985	1058
p-Xylene	931	994	1056
o-Xylene	958	1017	1099
m-Chlorotoluene	1033	1101	1190
p-Chlorotoluene	1035	1116	1194
o-Chlorotoluene	1026	1091	1178
m-Diethylbenzene	1089	1126	1228
p-Diethylbenzene	1102	1150	1235
o-Diethylbenzene	1106	1148	1255
trans-Decalin	1103	1127	1151
cis-Decalin	1143	1163	1210

TABLE 52

RETENTION INDICES OF SOME AROMATIC HYDROCARBONS MEASURED BY ENGEWALD *ET AL.*[339] ON OV-1 AND UCON LB-550X AT 100°C

Compound	*Retention index (i.u.)*	
	OV-1	*UCON LB-550X*
Benzene	663.7	759.2
Toluene	767.2	862.1
Ethylbenzene	859.1	950.3
Vinylbenzene	885.4	1008.7
n-Propylbenzene	949.5	1036.6
Isopropylbenzene	920.5	1004.4
n-Butylbenzene	1047.2	1134.0
Allylbenzene	938.6	1044.1
Isopropenylbenzene	972.6	1089.6

TABLE 53

RETENTION INDICES OF SOME ALKYLBENZENES ON SQUALANE AT 100°C MEASURED BY NABIVACH AND VASILIEV[365]

Compound	*Retention index (i.u.)*	*Compound*	*Retention index (i.u.)*
Benzene	651.1	*o*-Xylene	883.9
Toluene	757.9	1,3,5-Trimethylbenzene	967.9
Ethylbenzene	848.7	1,2,3-Trimethylbenzene	1011.6
p-Xylene	862.1	1,2,3,4-Tetramethylbenzene	1136.3
m-Xylene	864.1	Pentamethylbenzene	1260.5

TABLE 54

RETENTION INDICES OF SOME ALKYLBENZENES ON SQUALANE AT 100°C, AFTER MATISOVÁ[418]

Compound	*Retention index (i.u.)*					
	Ref. 480	*Ref. 481*	*Ref. 482*	*Ref. 483*	*Ref. 484*	*Ref. 485*
Benzene	648.0	650.4	647.9	649.2	650.5	651.1
Toluene	755.7	758.0	756.9	756.6	760.1	757.9
Ethylbenzene	846.1	847.7	847.7	846.9	850.0	848.7
1,4-Dimethylbenzene	860.0	861.8	862.9	860.8	864.6	862.2
1,3-Dimethylbenzene	861.5	863.2	865.7	862.7	864.8	864.1
1,2-Dimethylbenzene	882.5	883.7	885.6	882.9	886.0	883.9
Isopropylbenzene	905.6	908.0	907.1	906.3	908.4	907.7
n-Propylbenzene	934.7	936.1	936.2	935.4	938.0	936.1
1-Methyl-4-ethyl-benzene	950.0	951.3	950.8	950.6	954.3	951.3
1-Methyl-2-ethyl-benzene	963.1	964.7	966.5	963.9	966.0	964.3
1,3,5-Trimethylbenzene	966.8	967.6	969.8	967.5	969.0	967.9
1,2,4-Trimethylbenzene	984.7	988.2	–	985.5	987.0	986.3
1,2,3-Trimethylbenzene	1010.1	1011.9	1013.0	1011.1	1012.9	1011.6
tert.-Butylbenzene	972.3	973.3	973.2	972.9	971.7	973.5
sec.-Butylbenzene	988.6	989.8	989.5	989.3	990.2	990.2

TABLE 55

RETENTION INDICES OF SOME ALKYLBENZENES ON SE-30 AND OV-101 AT 100°C, AFTER MATISOVÁ[418]

Compound	*Retention index (i.u.)*		
	SE-30		*OV-101*
	Ref. 486	*Ref. 487*	
Benzene	661.8	663.1	663.6
Methylbenzene	764.4	765.9	766.4
Ethylbenzene	865.5	857.7	858.9
1,4-Dimethylbenzene	865.5	867.4	867.7
1,2-Dimethylbenzene	888.9	890.0	890.3
n-Propylbenzene	946.9	949.3	949.3
1-Methyl-3-ethylbenzene	956.6	956.2	955.9
1-Methyl-4-ethylbenzene	957.0	958.1	958.2
1,3,5-Trimethylbenzene	960.2	963.1	963.3
1-Methyl-2-ethylbenzene	973.4	973.3	972.8
1,2,4-Trimethylbenzene	984.7	985.9	988.4
tert.-Butylbenzene	985.9	989.0	987.6
sec.-Butylbenzene	1003.6	1006.0	1005.7
1,2,3-Trimethylbenzene	1018.9	1017.7	1015.9
1-Methyl-3-isopropylbenzene	1005.8	1013.2	1011.9
1-Methyl-4-isopropylbenzene	1012.0	1017.0	1017.8
1-Methyl-2-isopropylbenzene	1026.0	1028.4	1032.3
1-Methyl-3-*n*-propylbenzene	1041.4	1043.2	1043.4
1,3-Diethylbenzene	1042.4	1044.1	1040.4
1-Methyl-4-*n*-propylbenzene	1045.9	1047.1	1047.5
n-Butylbenzene	1046.5	1047.4	1048.1

TABLE 56

RETENTION INDICES OF SOME ALKYLBENZENES ON UCON LB-550X AND CITROFLEX A-4 STATIONARY PHASES AT 90°C, AFTER MATISOVÁ[418]

Compound	*Retention index (i.u.)*			
	UCON LB-550X		*Citroflex A-4*	
	Ref. 488	*Ref. 484*	*Ref. 483*	*Ref. 481*
Benzene	757.6	757.4	782.6	783.7
Ethylbenzene	948.8	947.7	975.8	975.7
1,4-Dimethylbenzene	959.9	957.8	982.6	982.8
1,3-Dimethylbenzene	963.6	961.1	986.1	985.7
1,2-Dimethylbenzene	995.1	988.5	1013.5	1013.0
Isopropylbenzene	1003.9	1001.2	1034.6	1033.7
1-Methyl-3-ethylbenzene	1047.2	1045.5	1072.7	1073.1
1-Methyl-4-ethylbenzene	1047.2	1045.5	1073.1	1072.9
1,3,5-Trimethylbenzene	1064.3	1063.4	1086.5	1082.7
1-Methyl-2-ethylbenzene	1068.1	1066.3	1094.8	1095.2
tert.-Butylbenzene	1069.7	1065.3	1101.9	1100.8
Isobutylbenzene	1080.7	1080.9	1111.4	1110.6
sec.-Butylbenzene	1084.7	1082.0	1114.7	1113.6
1,2,4-Trimethylbenzene	1087.6	1086.7	1110.1	1100.6
1-Methyl-3-isopropylbenzene	1094.4	1099.5	1126.6	1122.6
1,2,3-Trimethylbenzene	1120.8	1119.7	1143.7	1140.8
1,3-Diethylbenzene	1125.0	1123.1	1153.4	1153.4
1-Methyl-4-*n*-propylbenzene	1130.0	1131.6	1159.5	1158.0
1,4-Diethylbenzene	1133.3	1132.5	1162.9	1162.9
n-Butylbenzene	1134.8	1132.5	1162.8	1162.3
1-Methyl-2-*n*-propylbenzene	1144.1	1144.5	1174.5	1173.3

his results are presented in Table 63. Jánosi[118] determined retention indices of methylnaphthalenes on OV stationary phases (Table 64). Electronic and conformational effects of substituents on the retentions of *Z*/*E* isomeric olefins were rationalized by Priboth *et al.*[137].

Some interesting results for retention indices are given in Tables 65–70.

The retention behaviour of 32 synthesized C_6–C_{15} monoalkylcyclopentadienes on squalane and PEG-20M at 64°C was studied by Soják *et al.*[381] (Table 71). Specific interactions of alkanes with different metal ion forms of ion exchangers in GSC were studied by Hirsch *et al.*[17]. Tóth[87], using GLC and specific chemical reactions, analysed complex petrochemical mixtures and obtained excellent retention index values on SE-30 and PEG-20M stationary phases. Buchman *et al.*[103] studied the structure of substituted cyclohexenes on the basis of retention indices.

We also draw readers' attention to papers by Szederkényi *et al.*[144], Wu[155], Horna *et al.*[182], Kuchar *et al.*[198], Morishita *et al.*[206], Andersson[243], Komárek *et al.*[281], and Premecz and Ford[372]. Further useful results are summarized in Tables 72–76.

A further group of studies on retention index–molecular structure correlations are concerned with the determination of increments connected with different functional groups. Without trying to be exhaustive, of the many papers of this type we

TABLE 57

RETENTION INDICES OF SOME ALKYLBENZENES ON PEG-20M AND PEG-1540 STATIONARY PHASES AT 90°C, AFTER MATISOVÁ[418]

Compound	*Retention index (i.u.)*			
	PEG-20M			*PEG-1540 (ref. 285)*
	Ref. 488	*Ref. 484*	*Ref. 486*	
Benzene	933.4	941.3	960.3	960.2
Ethylbenzene	1111.1	1119.4	1141.3	1139.0
Isopropylbenzene	1154.9	1162.5	1186.3	1188.2
n-Propylbenzene	1190.3	1197.3	1220.5	1221.3
1,3,5-Trimethylbenzene	1220.3	1228.2	1253.6	1256.3
tert.-Butylbenzene	1215.1	1222.5	1237.5	1250.0
Isobutylbenzene	1215.9	1230.0	1251.7	1252.5
1,2,4-Trimethylbenzene	1253.1	1262.7	1289.0	1290.6
1,3-Diethylbenzene	1275.8	1282.1	1308.1	1308.0
1-Methyl-3-propylbenzene	1277.2	–	1310.0	1309.9
1-Methyl-4-propylbenzene	1279.9	1287.5	1310.8	1312.9
1,4-Diethylbenzene	1283.6	1281.3	1316.4	1316.6
n-Butylbenzene	1284.0	1293.4	1319.1	1318.6
1-Methyl-2-propylbenzene	1305.4	1312.9	1337.5	1339.7
sec.-Butylbenzene	1227.2	1234.6	1259.1	1260.3
1-Methyl-3-isopropylbenzene	1244.3	1253.0	1275.7	1275.1
1,2,3-Trimethylbenzene	1301.8	1311.0	1339.8	1340.0

can cite those by Haken *et al.*[65], Anders *et al.*[160], Peetre *et al.*[211], Szymanowski *et al.*[228], Haken and Korhonen[269,270] and Haken and Vernon[271].

The influence of increasing stationary phase polarity on the retention index increments of homologous aromatic esters, RCOOR′, where R and R′ represent the acid (acyl) and alcohol chains, respectively, was reported by Haken *et al.*[65]. Their

TABLE 58

RETENTION INDICES OF SOME ALICYCLIC HYDROCARBONS MEASURED BY ENGEWALD *ET AL.*[340] ON OV-1 AND UCON LB-550X AT 100°C

Compound	*Retention index (i.u.)*	
	OV-1	*UCON LB-550X*
Cyclooctane	927.2	957.7
Cyclononane	1035.7	1070.0
Cyclodecane	1133.9	1168.5
Cycloundecane	1225.4	1260.7
Cyclododecane	1323.7	1344.5
cis-Cyclododecane	1306.1	1356.0
trans-Cyclododecane	1291.5	1340.7

TABLE 59

RETENTION INDICES OF SOME CHLORINATED PHENYL ACETATES MEASURED BY HAKEN AND KORHONEN[61] ON SE-30 AND OV-351 AT 180°C

Compound	*Retention index (i.u.)*	
	SE-30	*OV-351*
Phenyl acetate	1025	1639
2-Chlorophenyl acetate	1192	1838
3-Chlorophenyl acetate	1219	1856
4-Chlorophenyl acetate	1224	1878
2,4- and 2,5-dichlorophenyl acetate	1351	1998
2,6-Dichlorophenyl acetate	1330	1985
2,3-Dichlorophenyl acetate	1384	2067
3,4-Dichlorophenyl acetate	1410	2086

TABLE 60

RETENTION INDICES OF CHLOROBENZENES MEASURED BY HAKEN AND KORHONEN[62] ON SE-30 AND PEG-20M AT 160°C

Compound	*Retention index (i.u.)*	
	SE-30	*PEG-20M*
Chlorobenzene	840	1270
1,3-Dichlorobenzene	1016	1434
1,4-Dichlorobenzene	1016	1471
1,2-Dichlorobenzene	1050	1514
1,3,5-Trichlorobenzene	1150	1545
1,2,4-Trichlorobenzene	1193	1653
1,2,3-Trichlorobenzene	1228	1735
1,2,3,5-Tetrachlorobenzene	1344	1786
1,2,4,5-Tetrachlorobenzene	1344	1793
1,2,3,4-Tetrachlorobenzene	1388	1908
Pentachlorobenzene	1525	1999
Hexachlorobenzene	1695	2178

TABLE 61

RETENTION INDICES OF SOME AROMATIC ESTERS MEASURED BY HAKEN *ET AL.*[65] ON DIFFERENT STATIONARY PHASES AT 200°C

Compound	*Retention index (i.u.)*			
	SE-30	*OV-25*	*QF-1*	*Silar-10C*
Benzyl acetate	1154	1450	1557	2330
Benzyl propionate	1245	1537	1620	2380
Benzyl butyrate	1337	1622	1700	2442
Benzyl hexanoate	1531	1807	1893	2618
Benzyl isobutyrate	1288	1557	1636	2330
Benzyl isopentanoate	1382	1651	1745	2437

TABLE 62

RETENTION INDICES OF SOME PERFLUOROCARBONS MEASURED BY MÜLLER *ET AL.*[79] ON DIFFERENT STATIONARY PHASES AT 50°C

Compound	*Retention index (i.u.)*				
	Squalane	*QF-1*	*PEG-1000*	*DEGS*	*OV-275*
Perfluoro-*n*-heptane	300	500	225	315	304
Perfluoro-*n*-octane	341	565	252	425	404
Perfluoro-*n*-decane	425	694	380	645	587
Perfluoro-*n*-undecane	473	771	473	768	668
Perfluoro-*n*-dodecane	528	857	558	877	772

TABLE 63

RETENTION INDICES OF VARIOUS COMPOUNDS MEASURED BY WINSKOWSKI[93] ON SE-30 AT 100°C AND ON OV-215 AT 80°C

Compound	*Retention index (i.u.)*	
	SE-30	*OV-215*
Methanol	384	550
Ethanol	427	618
1-Propanol	530	718
1-Butanol	637	827
1-Pentanol	754	–
Nitromethane	536	926
1-Nitropropane	712	1106
Pyridine	743	1009
Methyl ethyl ketone	579	888
2-Pentanone	671	980

TABLE 64

RETENTION INDICES OF SOME ALKYLNAPHTHALENES MEASURED BY JÁNOSI[118] ON DIFFERENT OV STATIONARY PHASES AT 150°C

Compound	*Retention index (i.u.)*					
	OV-1	*OV-3*	*OV-7*	*OV-11*	*OV-17*	*OV-25*
Naphthalene	1194	1235	1273	1330	1384	1471
1-Methylnaphthalene	1306	1348	1390	1450	1508	1602
2-Methylnaphthalene	1294	1335	1373	1430	1484	1571
2-Ethylnaphthalene	1382	1423	1461	1518	1572	1659
2-*n*-Propylnaphthalene	1470	1511	1549	1606	1660	1747
2-*n*-Butylnaphthalene	1570	1611	1649	1706	1760	1847

TABLE 65

RETENTION INDICES OF SOME 1-ALKENES AND *n*-ALKYLCYCLOPENTANES ON OV-1 AT 100°C, AFTER ANDERS *ET AL.*[160]

Compound	*Retention index (i.u.)*	*Compound*	*Retention index (i.u.)*
1-Butene	388.7	Propylcyclopentane	839.2
1-Pentene	488.8	Butylcyclopentane	938.3
1-Hexene	588.9	Pentylcyclopentane	1037.6
1-Heptene	689.0	Hexylcyclopentane	1137.9
1-Octene	789.0	Octylcyclopentane	1338.0
1-Nonene	888.7	Nonylcyclopentane	1438.0
1-Decene	988.6	Decylcyclopentane	1536.3
1-Undecene	1088.0	Undecylcyclopentane	1636.4
1-Dodecene	1187.9	Cyclohexane	673.6
1-Tetradecene	1387.9	Methylcyclohexane	734.6
Cyclopentane	574.5	Ethylcyclohexane	842.8
Methylcyclopentane	638.1	Propylcyclohexane	936.4
Ethylcyclopentane	743.4	Butylcyclohexane	1036.0

results gave retention plots with excellent regressions. As an example, the equation for phenyl esters on SE-30 stationary phase was

$$I = 957 + 96.5R \tag{19}$$

where R is the number of carbon atoms in the R groups, with a regression coefficient of 0.9999.

By application of the temperature coefficients of the retention index and the additivity principle of retention index increments, the structures of alkenylcycloalkanes and the positions of the double bonds were identified by Anders *et al.*[160]. Several equations for the calculation of the retention indices of alkylalkoxysilanes on Apiezon M and XE-60 at 160°C were given and discussed by Peetre *et al.*[211].

TABLE 66

RETENTION INDICES OF SOME PHENYLACETIC AND PHENYLPROPIONIC ACID DERIVATIVES MEASURED BY MACEK AND SMOLKOVÁ-KEULEMANSOVÁ[204] ON OV-101 AND OV-17 AT 200°C

Compound	*Retention index (i.u.)*	
	OV-101	*OV-17*
2-Phenylpropionic acid	1299.7	1453.0
Phenylacetic acid	1269.4	1442.8
4-Ethylphenylacetic acid	1452.2	1571.2
2-(4-Bromophenyl)propionic acid	1574.0	1751.3
4-Isopropoxy-3-chloro-phenylacetic acid	1766.5	1966.6

TABLE 67

RETENTION INDICES OF VARIOUS COMPOUNDS MEASURED BY SÁNCHEZ *ET AL.*[218] ON SQUALANE AND PEG-1540 AT 80°C

Compound	*Retention index (i.u.)*	
	Squalane	*PEG-1540*
Isopropyl formate	518	863
Methyl ethyl ketone	538	922
Ethyl acetate	548	900
sec.-Butanol	555	1041
Isobutanol	575	1109
n-Butanol	610	1166
Ethyl isobutyl ether	628	801
Diethyl ketone	640	997
Benzene	646	959
Cyclohexane	669	742
Ethyl *n*-butyl ether	669	863
Isooctane	693	675
Diisopropyl ketone	742	1020
Toluene	753	1057
Di-*n*-propyl ketone	817	1145

TABLE 68

RETENTION INDICES OF SOME ESTERS OF LOWER CARBOXYLIC ACIDS ON SP-400 AT 80°C, AFTER KOMÁREK *ET AL.*[281]

Compound	*Retention index (i.u.)*	*Compound*	*Retention index (i.u.)*
n-Propyl acetate	708.1	*n*-Butyl acetate	809.0
n-Propyl propionate	803.8	*n*-Butyl propionate	903.4
n-Propyl butyrate	893.2	*n*-Butyl butyrate	992.3
n-Propyl pentanoate	993.0	*n*-Butyl pentanoate	1088.9
n-Propyl hexanoate	1092.1	*n*-Butyl hexanoate	1189.0

TABLE 69

RETENTION INDICES OF SOME ALKYL ESTERS ON SP-400 AT 80°C, AFTER KOMÁREK *ET AL.*[281]

Ester	*Retention index (i.u.)*
Methyl propionate	626.8
Ethyl propionate	704.7
n-Propyl propionate	803.8
n-Butyl propionate	903.4
n-Pentyl propionate	1003.1

TABLE 70

RETENTION INDICES OF ETHYL 2,3-EPOXY-3-PHENYLPROPIONATE ON UCON 50-LB-550X STATIONARY PHASE AT DIFFERENT COLUMN TEMPERATURES MEASURED BY RIEGO AND GARCÍA-RASO[300]

Column temperature (°C)	*Retention index (i.u.)*
160	1352
180	1360
200	1370
220	1375
240	1390

GLC was used by Szymanowski *et al.*[228] to determine the polarity of pure model polyoxyethylene glycol dialkyl ethers and some of their sulphur analogues. The polarity parameters were correlated with the surfactant structure and increments for characteristic groups were determined at different column temperatures. Retention indices of halogenated derivatives of cyclohexane, benzene and anisole on Apolane-87 and PEG-20M at 150°C were reported by Haken and Vernon[271]. Retention increments due to the molecular structure and the size of the pendant groups together with the effect of increasing stationary phase polarity and concepts of additivity were discussed.

The most important aspect of the relationship between molecular structure and retention index is based on structural increments, the determination and application of which have been the subject of numerous reports, of which the contributions of the

TABLE 71

RETENTION INDICES OF SOME MONOALKYLCYCLOPENTADIENES MEASURED BY SOJÁK *ET AL.*[381] ON SQUALANE AND PEG-20M AT 64°C

Compound[a]	*Retention index (i.u.)*	
	Squalane	*PEG-20M*
1,3-CP	523.1	745.7
2-Methyl-1,3-CP	624.4	834.2
1-Methyl-1,3-CP	626.2	844.5
5-Methyl-1,3-CP	645.9	870.4
2-Ethyl-1,3-CP	723.8	930.3
1-Ethyl-1,3-CP	727.6	940.4
5-Ethyl-1,3-CP	747.6	966.7
2-*n*-Propyl-1,3-CP	812.6	1010.0
1-*n*-Propyl-1,3-CP	816.4	1020.2
5-*n*-Propyl-1,3-CP	835.1	1046.6
2-*n*-Butyl-1,3-CP	912.6	1108.2
1-*n*-Butyl-1,3-CP	916.5	1118.1
5-*n*-Butyl-1,3-CP	933.9	1144.5

[a] CP = cyclopentadiene.

TABLE 72

RETENTION INDICES OF SOME HYDROCARBONS MEASURED BY BERMEJO *ET AL.*[327] ON SQUALANE, OV-101, SE-54, OV-215 AND OV-210 AT 80°C

Compound	*Retention index (i.u.)*				
	Squalane	*OV-101*	*SE-54*	*OV-215*	*OV-210*
1,3-Cyclopentadiene	510.5	541.5	549.5	604.2	613.0
Cyclopentene	547.7	559.7	566.5	574.3	591.3
1-Methylcyclopentane	646.3	653.5	659.9	670.1	677.6
Cyclohexene	676.8	683.2	693.0	708.6	721.2
2,5-Norbornadiene	686.3	706.9	718.3	759.9	770.3
2-Norbornene	714.6	725.7	735.2	766.1	775.1
3-Methylcyclohexene	737.7	742.3	749.7	771.3	778.6
Methylenecyclohexane	737.8	744.5	754.1	779.0	791.0
4-Methylcyclohexene	739.9	744.3	751.2	771.4	785.8
1-Methylcyclohexene	768.5	771.2	780.4	793.0	802.7
Vinylcyclohexane	821.4	823.2	831.9	861.9	869.9
4-Vinylcyclohexene	825.1	832.8	843.6	875.4	885.8
Ethylidenecyclohexane	861.8	868.8	878.0	895.6	905.3
cis-Cyclodecene	1111.3	1112.6	1126.5	1169.9	1183.5

groups of Bata[1,41,97,147], Boneva[5,43,44,249–253,259,329,330,407], Brooks[45,46,102a,331,402,403], Calixto[8,9,51,52,104,104a,138,170a] and Castello[10,53,105,257,404] and those listed in the Introduction can be considered as the most valuable sources.

After having completed studies on the relationship between structure and retention index increments for normal and isomeric alkanes[451,452], our group developed a method that made it possible to study the C–H bonds in the methyl groups in alkanes[385]. The fundamental equations in the calculation method were discussed in our previous review[47] (eqns. 74, 82 and 90–99).

TABLE 73

RETENTION INDICES OF SOME ALKYLQUINOLINES MEASURED BY BURYAN *ET AL.*[332] ON DIFFERENT STATIONARY PHASES AT 160°C

Quinoline[a]	*Retention index (i.u.)*		
	OV-101	*UCON LB-550X*	*PEG-20M*
Q	1246	1478	1924
IsoQ	1269	1503	1958
2-MQ	1308	1527	1946
8-MQ	1319	1527	1943
7-MQ	1354	1581	2020
6-MQ	1358	1582	2020
3-MQ	1361	1590	2042
2,8-M_2Q	1374	1563	1945
4,6-M_2Q	1487	1712	2159
2,6,8-M_3Q	1475	1667	2042

[a] Q = quinoline; M = methyl.

TABLE 74

RETENTION INDICES OF SOME CYCLOHALOGENS MEASURED BY EVANS AND HAKEN[341] ON APOLANE-87 AND PEG-20M AT 150°C

Compound	*Retention index (i.u.)*	
	Apolane-87	*PEG-20M*
Benzene	687	971
Fluorobenzene	680	996
Chlorobenzene	880	1231
Bromobenzene	974	1351
Iodobenzene	1088	1504
Anisole	928	1340
Fluoroanisole	916	1367
Chloroanisole	1120	1592
Bromoanisole	1212	1706
Iodoanisole	1326	1852
Cyclohexane	680	740
Fluorocyclohexane	735	990
Chlorocyclohexane	918	1173
Bromocyclohexane	1009	1288
Iodocyclohexane	1117	1405

The bond energies are used as a basis for calculation of the bond index increments. Bond energy data, E (kJ), can be found in the literature or calculated from equations as follows:

$$E_{C-H} = 45.27/d \tag{20}$$

TABLE 95

COMPARISON OF RETENTION INDICES OF ALKYLALKOXYSILANES MEASURED AND CALCULATED BY PEETRE *ET AL.*[211] ON APIEZON M AT 160°C

Compound	*Retention index (i.u.)*			
	160°C			*180°C on SE-54*
	OV-17	*OV-215*	*PEG-20M*	
α-Ribofuranose	1624	1675	1528	1626
β-Ribofuranose	1647	1688	1550	1636
α-Ribopyranose	1651	1775	1550	1663
β-Ribopyranose	1626	1709	1531	1642
α-Arabinofuranose	1597	1609	1531	1596
β-Arabinofuranose	1682	1738	1603	1666
α-Arabinopyranose	1597	1690	1524	1601
β-Arabinopyranose	1643	1717	1586	1635

TABLE 76

RETENTION INDICES OF SOME NON-IONIC SURFACTANTS MEASURED BY VOELKEL[387] ON DIFFERENT STATIONARY PHASES AT 170°C

Basic structure: $RX(CH_2CH_2O)_nR'$.

R	*R'*	*X*	*n*	*Retention index (i.u.)*		
				Apiezon K	*SE-30*	*QF-1*
C_4H_9	H	O	0	675	675	867
	H	O	2	1150	1193	1469
	H	O	3	1437	1479	1797
C_6H_{13}	H	S	0	931	918	1043
	H	S	1	1318	1317	1621
	H	S	2	1564	1578	1952
	H	S	3	1829	1844	2273
	H	O	0	815	822	1003
	H	O	1	1054	1090	1534
	H	O	2	1325	1363	1662
	H	O	3	1576	1631	1983
	H	NH	0	815	829	993
	H	NH	1	1183	1197	1475
	H	NH	2	1424	1443	1757
	H	NH	3	1656	1703	2082
C_8H_{17}	H	S	0	1200	1140	1298
	H	S	1	1603	1562	1918
	H	S	2	1971	1830	2277
	H	S	3	2045	2123	2604
	H	O	0	1044	999	1299
	H	O	1	1274	1282	1569
	H	O	2	1552	1568	1928
	H	O	3	1822	1855	2272
	H	NH	0	1091	993	1230
	H	NH	1	1408	1333	1711
	H	NH	2	1645	1608	2062
	H	NH	3	1913	1917	2355
C_6H_{13}	CH_3	O	3	1592	1660	1903
C_8H_{17}	CH_3	O	3	1837	1880	2175
$C_{10}H_{21}$	CH_3	O	3	2028	2121	2435

and

$$E_{C-C} = 53.56/d \tag{21}$$

where C–H denotes the carbon–hydrogen bond in alkanes, C–C the carbon–carbon bond in alkanes and d is the bond distance (nm). The bond distance can be approximated by

$$d = 0.819 \sum_{i=1}^{2} a_0(i) + 0.157 \sum_{j=1}^{p} a_1(j) + 0.024 \sum_{k=1}^{q} a_2(k) \tag{22}$$

where

i = serial number of the atoms in the bond examined;
j = serial number of the atoms of the bond in the primary environment;
k = serial number of the atoms of the bond in the secondary environment;
p = total number of atoms of the bond in the primary environment;
q = total number of atoms of the bond in the secondary environment;
0.819 = bond factor;
0.157 = primary environment factor;
0.024 = secondary environment factor;
a_0 = the atomic code number in the bond;
a_1 = the atomic code number in the primary environment;
a_2 = the atomic code number in the secondary environment [the atomic code numbers of alkanes are single-digit numbers (see Table 77)].

The calculation of the bond index increments from the bond distances is based on following equations:

$$i_b(\text{C–H bonds}) = 0.815/d \tag{23}$$

$$i_b(\text{C–C bonds}) = 0.964/d \tag{24}$$

Coding rules for the bonds in alkanes are summarized in the original paper[385].

Similarly to the case of the retention index increments of alkanes, the molecular structures of benzene and alkylbenzenes can be coded and described[77a,510]. For example, let us calculate the relevant data for benzene. The retention index of benzene on OV-3 stationary phase at 80°C is 681.9 i.u.[55], the C–C bond length is 0.1397 nm with a bond energy of 119.86 kcal and the C–H bond length is 0.1084 nm with a bond energy of 99.82 kcal[454,455]. Thus, the formation (atomization) energy of benzene is 1318.08 kcal/mol. The molecular structural coefficient of benzene on OV-3 at 80°C can be calculated as follows:

$$S^{\text{OV-3}}_{c,\text{benzene}}(80°\text{C}) = \frac{100}{b}\log V_g = I - K_c = 681.9 - 149.1 = 532.8\ \{\log [\text{cm}^3(\text{He})/\text{g(OV-3)}]\} \tag{25}$$

TABLE 77

BOND LENGTH INCREMENT VALUES OF ALKANES AND BOND CODES

Atom	*Bond code*	*Bond length increment value (nm)*
Primary carbon	1	0.07900
Secondary carbon	2	0.06500
Tertiary carbon	3	0.05565
Quaternary carbon	4	0.04978
Hydrogen bound to primary carbon	5	0.02700
Hydrogen bound to secondary carbon	6	0.03100
Hydrogen bound to tertiary carbon	7	0.03500

where S_c is the molecular structural coefficient: log [cm^3(carrier gas)/g(stationary phase); K_c is the Kováts coefficient {− log [cm^3(carrier gas)/g(stationary phase)]};

$$K_c^{OV\text{-}3}(80°C) = 149.1 \{- \log [cm^3(He)/g(OV\text{-}3)]\} \quad (26)$$

The bond index increments are calculated in i.u. from the bond energy using the factor[385]

$$f_c = 0.0513 \text{ i.u./kcal.} \quad (27)$$

From S_c:

$$f_S = \frac{\text{molecular structural coefficient}}{\text{formation (atomization) energy}} \cdot$$

$$\left(\frac{\log [cm^3(\text{carrier gas})/g(\text{stationary phase})] \text{ mol}}{\text{kcal}}\right) \quad (28)$$

Structural increments are calculated in log [cm^3(carrier gas)/g(stationary phase)] mol from the bond energy by f_S as follows:

$$f^{OV\text{-}3}_{S,\text{benzene}}(80°C) = 532.8/1318.08$$
$$= 0.4042 \log [cm^3(\text{carrier gas})/g(\text{stationary phase})] \text{ mol/kcal} \quad (29)$$

Hence

$$i_b(C\text{–}C) = 119.86 \cdot 0.0513 = 6.15 \text{ i.u.} \quad (30)$$

$$i_b(C\text{–}H) = 99.82 \cdot 0.0513 = 5.12 \text{ i.u.} \quad (31)$$

where i_b = bond index increment (i.u.).

$$i_{s1}^{OV\text{-}3}(80°C) = 119.86 \cdot 0.4042$$
$$= 48.45 \log [cm^3(\text{carrier gas})/g(\text{stationary phase})] \text{ mol} \quad (32)$$

$$i_{s2}^{OV\text{-}3}(80°C) = 99.82 \cdot 0.4042$$
$$= 40.35 \log [cm^3(\text{carrier gas})/g(\text{stationary phase})] \text{ mol} \quad (33)$$

for two different bonds in benzene, where i_s = structural increment log [cm^3(carrier gas)/g(stationary phase)] mol. Atomic code numbers[47] of benzene and alkylbenzenes are summarized in Table 78.

The application of bond increment values gives a new possibility for studying the relationship between molecular structure and retention index. The effect of the primary and secondary atomic environments on the interactions of some alkane C–H bonds on a squalane stationary phase at different column temperatures was studied by Tekler *et al.*[385] and the results are given in Table 79.

Research based on structural increments is expected to make significant progress in the future. Development will be extremely rapid in those fields in which, in addition to literature sources, one can set up additional relationships which provide the

TABLE 78

BOND LENGTH INCREMENT VALUES OF BENZENE AND METHYLBENZENES AND BOND CODES

Atom	*Bond code*	*Bond length increment value (nm)*
Carbon in the methyl group	A1	0.08755
Hydrogen in the methyl group	A2	0.02360
Tertiary carbon in the ring	A3	0.06267
Quaternary carbon in the ring	A4	0.05240
Hydrogen bound to ring	A5	0.03966

TABLE 79

INTERACTION INDEX INCREMENTS OF SOME ALKANE C–H BONDS ON SQUALANE STATIONARY PHASE AT DIFFERENT COLUMN TEMPERATURES AND FOR DIFFERENT ATOMIC ENVIRONMENTS, AFTER TEKLER *ET AL.*[385]

Hydrogen was used as the carrier gas. C_3 = propane; C_4 = butane; C_5 = pentane; M = methyl; E = ethyl.

Compound	*Bond code*	*Interaction index increment*				
		30°C	*50°C*	*70°C*	*100°C*	*120°C*
Primary C–H bonds						
CH_4	[C–H]	17.12	17.12	17.12	17.12	17.12
$22M_2C_3$	15:4 (111) 55; 0.	19.01	19.08	19.16	19.29	19.39
C_2H_6	15:1 (555) 55; 0.	21.37	21.37	21.37	21.37	21.37
$2MC_3$	15:3 (117) 55; 0.	21.53	21.57	21.60	21.62	21.63
$22M_2C_4$	15:4 (112) 55; 0.	21.75	21.84	21.93	22.06	22.15
	15:2 (466) 55; 0.	21.80	21.89	21.98	22.11	22.20
$223M_3C_4$	15:4 (113) 55; 0.	22.65	22.80	22.95	23.17	23.32
	15:3 (147) 55; 0.	22.70	22.85	23.00	23.22	23.37
C_3H_8	15:2 (166) 55; 0.	23.28	23.28	23.28	23.28	23.28
$2233M_4C_4$	15:4 (114) 55; 0.	22.60	22.82	23.04	23.37	23.59
$2MC_4$	15:2 (366) 55; 0.	23.25	23.30	23.35	23.41	23.44
	15:3 (127) 55; 0.	23.25	23.30	23.35	23.41	23.44
$2234M_4C_5$	15:3 (347) 55; 0.	23.15	23.32	23.49	23.75	23.92
$23M_2C_4$	15:3 (137) 55; 0.	23.46	23.58	23.70	23.90	24.04
$223M_3C_4$	15:3 (247) 55; 0.	23.65	23.79	23.92	24.12	24.25
n-C_4H_{10}	15:2 (266) 55; 0.	24.29	24.29	24.29	24.29	24.29
$234M_3C_5$	15:3 (337) 55; 0.	24.32	24.45	24.58	24.75	24.86
$33M_2C_5$	15:4 (122) 55; 0.	24.35	24.49	24.64	24.88	25.06
$3MC_5$	15:3 (227) 55; 0.	24.68	27.74	24.81	24.92	25.00
$23M_2C_5$	15:3 (237) 55; 0.	24.99	25.09	25.20	25.32	25.40
$22334M_5C_5$	15:4 (134) 55; 0.	24.85	25.13	25.41	25.83	26.11
$233M_3C_5$	15:4 (123) 55; 0.	25.20	25.38	25.57	25.84	26.03
$2233M_4C_5$	15:4 (124) 55; 0.	25.66	25.87	26.07	26.36	26.54
$2334M_4C_5$	15:4 (133) 55; 0.	26.00	26.21	26.41	26.71	26.91
$3M3EC_5$	15:4 (222) 55; 0.	27.16	27.18	27.21	27.25	27.28
$23M_23EC_5$	15:4 (223) 55; 0.	27.38	27.59	27.79	28.10	28.31
$223M_33EC_5$	15:4 (224) 55; 0.	27.66	27.94	28.22	28.66	28.96
$234M_33EC_5$	15:4 (233) 55; 0.	27.84	28.10	28.35	28.72	28.96

(*Continued on p. 48*)

TABLE 79 (*continued*)

Compound	*Bond code*	*Interaction index increment*				
		30°C	*50°C*	*70°C*	*100°C*	*120°C*
Secondary C–H bonds						
$22M_2C_4$	26:1 (555) 4 (111) 6; 0.	21.19	21.27	21.36	21.49	21.58
C_3H_8	26:1 (555) 1 (555) 6; 0.	22.60	22.60	22.60	22.60	22.60
$2MC_4$	26:1 (555) 3 (117) 6; 0.	22.66	22.71	22.75	22.81	22.84
$223M_3C_5$	26:1 (555) 3 (147) 6; 0.	23.04	23.18	23.31	23.50	23.62
n-C_4H_{10}	26:1 (555) 2 (166) 6; 0.	23.67	23.67	23.67	23.67	23.67
$33M_2C_5$	26:1 (555) 4 (112) 6; 0.	23.71	23.85	23.99	24.23	24.40
$3MC_5$	26:1 (555) 3 (127) 6; 0.	24.05	24.11	24.17	24.28	24.36
$23M_2C_5$	26:1 (555) 3 (137) 6; 0.	24.34	24.44	24.53	24.66	24.74
$233M_3C_5$	26:1 (555) 4 (113) 6; 0.	24.55	24.73	24.90	25.17	25.35
$2233M_4C_5$	26:1 (555) 4 (114) 6; 0.	24.99	25.19	25.39	25.67	25.85
$3M3EC_5$	26:1 (555) 4 (122) 6; 0.	26.45	26.48	26.50	26.54	26.57
$23M_23EC_5$	26:1 (555) 4 (123) 6; 0.	26.67	26.87	27.07	27.37	27.57
$223M_33EC_5$	26:1 (555) 4 (124) 6; 0.	26.93	27.21	27.49	27.91	28.20
Tertiary C–H bonds						
$2MC_3$	37:1 (555) 1 (555) 1 (555); 0.	19.95	19.99	20.01	20.03	20.04
$223M_3C_4$	37:1 (555) 1 (555) 4 (111); 0.	21.05	21.19	21.33	21.54	21.68
$2MC_4$	37:1 (555) 1 (555) 2 (166); 0.	21.61	21.66	21.70	21.76	21.79
$2234M_4C_5$	37:1 (555) 3 (117) 4 (111); 0.	21.54	21.70	21.86	22.10	22.26
	37:1 (555) 1 (555) 3 (147); 0.	21.50	21.66	21.82	22.06	22.22
$23M_2C_4$	37:1 (555) 1 (555) 3 (117); 0.	21.82	21.92	22.04	22.22	22.35
$223M_3C_5$	37:1 (555) 2 (166) 4 (111); 0.	22.01	22.14	22.27	22.45	22.57
$234M_3C_5$	37:1 (555) 1 (555) 3 (137); 0.	22.60	22.73	22.84	23.00	23.10
	37:1 (555) 3 (117) 3 (117); 0.	22.68	22.80	22.92	23.08	23.18
$3MC_5$	37:1 (555) 2 (166) 2 (166); 0.	23.03	23.08	23.14	23.25	23.33
$23M_2C_5$	37:1 (555) 1 (555) 3 (127); 0.	23.22	23.32	23.41	23.53	23.60
	37:1 (555) 2 (166) 3 (117); 0.	23.30	23.40	23.49	23.61	23.68
$22334M_5C_5$	37:1 (555) 1 (555) 4 (114); 0.	23.09	23.35	23.61	24.00	24.26
$233M_3C_5$	37:1 (555) 1 (555) 4 (112); 0.	23.42	23.59	23.76	24.01	24.18
$2334M_4C_5$	37:1 (555) 1 (555) 4 (113); 0.	24.16	24.35	24.54	24.82	25.01
$23M_23EC_5$	37:1 (555) 1 (555) 4 (122); 0.	25.45	25.64	25.83	26.12	26.31
$234M_33EC_5$	37:1 (555) 1 (555) 4 (123); 0.	25.86	26.10	26.33	26.68	26.91

necessary data (bond distance and energy, specific retention volume, etc.) for calculation of the structural increments.

For further information about molecular structure–retention index relationships, see refs. 1, 2, 4, 5, 19a, 23, 25, 27, 28, 35a, 35b, 36, 37a, 39a, 40a, 41, 45, 54, 55, 71, 75, 91, 92, 95, 94, 97, 109, 114, 115, 118, 126, 136, 151, 158, 159, 170, 181, 197, 201, 204, 208, 210, 212, 213, 219, 226, 232, 236, 238, 246, 247, 250, 257, 265, 285, 287a, 292, 295–297, 300, 309, 327, 330–333, 337, 344, 361, 363, 364, 366, 374, 382, 384, 398, 403, 410, 414, 415, 417, 421, 424a, 428, 433 and 475.

10. ROHRSCHNEIDER'S CONCEPT AND CALCULATION METHOD

As nearly all the important questions pertaining to the Rohrschneider concept and calculation method were been dealt with in our previous review[47], here we discuss

only the newest results in this very significant area. In recent years researchers seem to have focused on the problems of the precalculation of retention indices.

The ΔI value can be calculated by Rohrschneider's equation in classical GLC. For example, let us calculate the ΔI value of pyridine on PEG-4000 at 120°C by Rohrschneider's equation from McReynolds constants[439]. We can write the following system of equations:

$$\text{on Apiezon L: } 42 = 0.13a^R + 0.35b^R + 0.11c^R + 0.31d^R + 0.33e^R \quad (34)$$
$$\text{on OV-3: } 88 = 0.55a^R + 0.39b^R + 0.46c^R + 0.84d^R + 0.17e^R \quad (35)$$
$$\text{on OV-17: } 202 = 1.12a^R + 1.19b^R + 1.05c^R + 1.84d^R + 0.69e^R \quad (36)$$
$$\text{on PEG-20M: } 510 = 3.87a^R + 2.82b^R + 2.21c^R + 4.34d^R + 1.48e^R \quad (37)$$
$$\text{on DEGS: } 860 = 5.95a^R + 4.22b^R + 3.23c^R + 7.25d^R + 2.40e^R \quad (38)$$

where a^R, b^R, c^R, d^R and e^R = substance-specific factors according to Rohrschneider[47]. On solving this system of equations the values $a^R = -18.509$, $b^R = 150.997$, $c^R = -138.944$, $d^R = 146.524$ and $e^R = -116.914$ were obtained.

Using these substance-specific factors and McReynolds constants on PEG-4000 at 120°C, we can write

$$\Delta I^{\text{PEG-4000–squalane}}_{\text{pyridine}}(120°C) = (3.99 \cdot -18.509) + (2.85 \cdot 150.997) +$$
$$+ (2.24 \cdot -138.944) + (4.43 \cdot 146.524) + (1.48 \cdot -116.914) = 521.3 \text{ i.u.} \quad (39)$$

The value as measured by McReynolds[439] was 520.0 i.u.

It could also be established from Rohrschneider's paper[44] that the minimum number of standard substances is three and the optimum is five. As an example, the calculation of the interaction index increment of the C–H bond [37:1 (555) 1 (555) 1 (555); 0.] (for an explanation of the code, see ref. 385) on squalane stationary phase at 100°C with three standard substances is performed using the following system of equations:

$$19.95 = 21.05s_1 + 21.61s_2 + 21.54s_3 \quad (40)$$
$$20.01 = 21.33s_1 + 21.70s_2 + 21.86s_3 \quad (41)$$
$$20.04 = 21.68s_1 + 21.79s_2 + 22.26s_3 \quad (42)$$

On solving these equations the values $s_1 = -6.90$, $s_2 = 2.00$ and $s_3 = 5.66$ were obtained.

Using s_i values and suitable data from the paper of Tekler *et al.*[385], we can write

$$i_i^{Sq}(100°C) = (21.54 \cdot -6.90) + (21.76 \cdot 2.0) + (22.10 \cdot 5.66) = 19.98 \text{ i.u.} \quad (43)$$

where i_i is the interaction index increment value (i.u.)[385]. The increment value measured was 20.03 i.u.

Recently, our group has come to the conclusion that those papers by Rohrschneider which deal with specific retention volume are at least as important as those connected with the retention index, which prompted us to begin extensive research in this field. Early results of these investigations are summarized in Tables 2–6. The basic ideas in our calculation method are presented below for the case of

cis-3-methyl-2-pentene. From the papers by Chien *et al.*[54,55], the following equation systems can be written:

System I: $\log V_g$ values

Guiding principle:

$$\log V_g(s) = \log V_g(n\mathrm{C}_5) \cdot s_1 + \log V_g(n\mathrm{C}_6) \cdot s_2 + \log V_g(n\mathrm{C}_7) \cdot s_3 + \\ + \log V_g(n\mathrm{C}_8) \cdot s_4 + \log V_g(n\mathrm{C}_9) \cdot s_5 \quad (44)$$

where V_g = specific retention volume [cm^3 (carrier gas)/g(st.ph.)], s = substance examined, s_i = substance-specific factor and $n\mathrm{C}_z$ = *n*-alkane with carbon number z. Then,

$$\text{on OV-1: } 97.48 = 36.87s_1 + 88.47s_2 + 211.4s_3 + 504.5s_4 + 1202s_5 \quad (45)$$
$$\text{on OV-3: } 90.97 = 32.15s_1 + 79.29s_2 + 195.6s_3 + 482.3s_4 + 1190s_5 \quad (46)$$
$$\text{on OV-7: } 85.15 = 28.56s_1 + 71.26s_2 + 176.3s_3 + 437.9s_4 + 1090s_5 \quad (47)$$
$$\text{on OV-17: } 62.89 = 18.94s_1 + 46.60s_2 + 114.10s_3 + 282.50s_4 + 693.60s_5 \quad (48)$$
$$\text{on OV-25: } 41.28 = 11.60s_1 + 28.15s_2 + 68.36s_3 + 165.80s_4 + 405.90s_5 \quad (49)$$

On solving these equations the values $s_1 = -5.743$, $s_2 = 1.939$, $s_3 = 5.067$, $s_4 = 7.313$ and $s_5 = -7.901$ at 50°C were obtained. Using these s_i values and suitable data from the papers by Chien *et al.*[54,55], we can write

$$\log V_g^{\text{OV-11}}(50^\circ\mathrm{C}) = (23.26 \cdot -5.743) + (57.78 \cdot 1.939) + (143.4 \cdot 5.067) + \\ + (355.9 \cdot 7.313) + (883.3 \cdot -7.901) = 1.87407 \quad (50)$$

Hence $V_g^{\text{OV-11}}(50^\circ\mathrm{C})_{\text{calculated}}$ = antilog 1.87407 = 74.83 cm^3 He/g OV-11. The specific retention volume measured was 73.28 cm^3 He/g OV-11.

System II: $\log V_g(s)^{\text{st.ph.}}/\log V_g(s)^{\text{OV-1}}$ values

Guiding principle:

$$\frac{\log V_g(s) \text{ on st.ph. } i}{\log V_g(s) \text{ on OV-1}} = \sum_{i=1}^{5} \left[\frac{\log V_g(n\mathrm{C}_{i+4})^{\text{st.ph.}}}{\log V_g(n\mathrm{C}_{i+4})^{\text{OV-1}}} \right] \cdot s_i \quad (51)$$

$$\text{OV-3/OV-1: } 0.985 = 0.962s_1 + 0.976s_2 + 0.985s_3 + 0.993s_4 + 0.999s_5 \quad (52)$$
$$\text{OV-7/OV-1: } 0.970 = 0.929s_1 + 0.952s_2 + 0.966s_3 + 0.977s_4 + 0.986s_5 \quad (53)$$
$$\text{OV-11/OV-1: } 0.938 = 0.872s_1 + 0.905s_2 + 0.928s_3 + 0.944s_4 + 0.957s_5 \quad (54)$$
$$\text{OV-17/OV-1: } 0.904 = 0.815s_1 + 0.857s_2 + 0.885s_3 + 0.907s_4 + 0.922s_5 \quad (55)$$
$$\text{OV-25/OV-1: } 0.812 = 0.679s_1 + 0.745s_2 + 0.789s_3 + 0.821s_4 + 0.847s_5 \quad (56)$$

On solving these equations the values $s_1 = -0.928$, $s_2 = 1.441$, $s_3 = -2.024$, $s_4 = 6.000$ and $s_5 = -3.491$ were obtained. The sum of substance-specific factors can be calculated as follows:

$$\sum_{i=1}^{5} s_i = -0.928 + 1.441 - 2.024 + 6.0 - 3.491 = 0.998 \quad (57)$$

The theoretical value[47] of the sum of substance-specific factors is 1.000.

Some Rohrschneider constants are given in Tables 80 and 81.

11. McREYNOLDS' SYSTEM. POLARITY OF STATIONARY PHASES

In recent years it has been established that of the ten McReynolds constants[439] the first five (for benzene, *n*-butanol, 2-pentanone, nitropropane and pyridine) are sufficient for both research and everyday practice.

Widespread use of the McReynolds system is hindered partly by some erroneously measured data, and especially by the fact that these constants were determined only at 120°C. Combination of the Rohrschneider[441] and McReynolds[439] systems supplemented with data measured at 80 and 140°C would ensure greater development of this area in the future. The question of the polarity of stationary phases is also being studied extensively and various aspects of this problem have been dealt with by many investigators in recent years.

TABLE 80

ROHRSCHNEIDER CONSTANTS OF SOME STATIONARY PHASES USED IN GLC, AFTER HAKEN AND KHEMANGKORN[442] AND CSIZMADIÁNÉ[443]

Stationary phase	*Rohrschneider constant*				
	x	*y*	*z*	*u*	*s*
F-61	0.31	0.49	0.82	1.08	0.83
85% Phenyl	1.88	2.33	2.15	3.80	2.95
Hallcomid M-18-OL	0.96	2.93	1.59	2.99	1.77
Citroflex A-4	1.36	2.66	2.11	3.70	2.33
Zonyl E-7	2.37	4.37	5.16	5.84	–
Hyprose SP-80	2.96	5.57	4.22	6.53	5.91
Diethylene glycol succinate (DEG 5)	4.93	7.58	6.14	9.50	8.37
PEG-5000 K_2	2.69	5.11	3.38	6.53	4.40

TABLE 81

RETENTION INDICES OF ROHRSCHNEIDER STANDARDS ON OV-225 AT 90°C, AFTER ISENBERG *ET AL.*[117]

Compound	*Retention index (i.u.)*
Benzene	854
Butanone	705
Ethanol	911
Nitromethane	968
Pyridine	1068

Vigdergauz *et al.*[464] developed a technique for the classification of liquid crystalline stationary phases for the GC analysis of positional isomers. The influence of the polarity of stationary phases on the retention of solutes with different molecular structures was studied by Calixto and Raso[9].

A compilation of solubility data for 240 substances on 207 stationary phases was published by Patte *et al.*[28a]. Correlation between polarity of stationary phases and the retention data of solutes with various numbers of methylene groups was examined by Sidorov[34a] and Stolyarov and Kartsova[35c]. An optimization method for selecting stationary phases was described by Vigdergauz and Bankoskaya[38a]. A classification of the "polarity" of porous polymer bead stationary phases by comparison with squalane and Apolane-87 was given by Castello and D'Amato[53]. Markides *et al.*[76,77] studied cyanosilicones as stationary phases in GLC.

Fernandez-Sanchez *et al.*[142] obtained interesting results using McReynolds constants relative to *n*-decane. The interactions involved were studied with the help of Snyder's selectivity triangle, using the retention indices and the selectivity parameters[220,248]. A procedure was described by Krupčik *et al.*[286] for the computer optimization of the selectivity of capillary columns in series for the GC separation of cyclic and aromatic hydrocarbons. Useful results on siloxane–silarylene copolymer stationary phases were presented by Bemgård *et al.*[322]. A new method of classification was applied to 233 stationary phases used in GLC by García-Dominguez *et al.*[336]. A classification of GC stationary phases by a new variant of the Kováts retention index system was reported by Evans and Haken[342].

Solute–solvent interactions in liquid alkylammonium-4-toluenesulphonate salts were studied by Furton and Poole[345]. GC stationary phase properties of two room-temperature liquid organic salts were demonstrated with interesting results by Furton *et al.*[346]. Two valuable methods were used by Kersten and co-workers[354,355] to establish the influence of concurrent retention mechanisms (GSC and GLC) on the

TABLE 82

McREYNOLDS STANDARDS OF SOME LIQUID CRYSTALLINE STATIONARY PHASES MEASURED BY SZULC AND WITKIEWICZ[85] AT 120°C

Symbol[a] *of stationary phase*	*Retention index (i.u.)*				
	Benzene	*n-Butanol*	*2-Pentanone*	*1-Nitopropane*	*Pyridine*
A_1	809	858	860	980	1010
A_2	802	848	855	966	1016
A_3	884	936	929	1086	1103
A_4	894	918	942	1079	1174
A_5	838	891	891	1019	1053
B_1	803	815	835	927	1019
B_2	831	853	863	980	1036
B_3	824	825	844	937	1019
C_1	858	910	913	1049	1081
C_2	838	866	888	1021	1063
C_3	837	844	852	985	1023

[a] Symbols as in original paper[85].

TABLE 83

RETENTION INDICES OF McREYNOLDS STANDARDS MEASURED AND CALCULATED BY FERNANDEZ-SANCHEZ *ET AL.*[379] ON DIFFERENT STATIONARY PHASES AT 120°C

McReynolds standard	*Retention index (i.u.)*				
	OV-101	*OV-25*	*PEG-20M*	*OV-225*	*SP-2340*
Benzene	672	828	992	871	1189
n-Butanol	663	796	1178	951	1362
2-Pentanone	685	840	1053	957	1262
1-Nitropropane	724	954	1279	1130	1586
1-Iodobutane	821	986	1111	1035	1287
cis-Hydrindane	1004	1117	1156	1115	1246

accuracy of McReynolds constants. Interesting results were presented by Pomaville and Poole[369] on thermally stable, highly fluorinated stationary phases in GLC. Poole *et al.*[371,424], re-examined Rohrschneider and McReynolds constants. The polarity of OV and SE series stationary phases was estimated by García-Raso *et al.*[375]. Some McReynolds constants on different stationary phases at different temperatures are summarized in Tables 82–87.

Complementing the polarity table published in our previous review[47], the retention polarity values of the most frequently used stationary phases, mostly based on McReynolds constants obtained at 120°C[439], are summarized in Table 88.

For further information, see also refs. 52, 79, 117, 140, 143a, 162, 263, 299, 350, 351, 352, 370, 423, 388, 389, 406 and 425.

11.1. Mixed stationary phases

Researchers working in different areas of GLC soon realized the significance of using mixed stationary phases (tandem, mixed stationary phases, mixing packings,

TABLE 84

RETENTION INDICES OF ROHRSCHNEIDER AND McREYNOLDS STANDARDS MEASURED BY CASTELLO AND D'AMATO[53] ON SQUALANE AT DIFFERENT COLUMN TEMPERATURES

Compound	*Retention index (i.u.)*	
	100°C	*125°C*
Ethanol	396	391
Methyl ethyl ketone	533	534
Nitromethane	467	463
Benzene	647	652
n-Butanol	588	590
2-Pentanone	624	626
Pyridine	707	711
1-Nitropropane	647	652

TABLE 85

RETENTION INDICES OF ROHRSCHNEIDER AND McREYNOLDS STANDARDS MEASURED BY CASTELLO AND D'AMATO[53] ON APOLANE-87 AT DIFFERENT COLUMN TEMPERATURES

Compound	*Retention index (i.u.)*	
	100°C	*200°C*
Ethanol	413	397
Methyl ethyl ketone	541	542
Nitrometane	500	500
Benzene	670	702
n-Butanol	603	610
Methyl propyl ketone	630	636
Pyridine	719	757
1-Nitropropane	662	678

etc.) and this idea led to important results from both theoretical and practical points of view.

There is steadily growing interest in this field even now, in spite of the fact that the development and marketing of a standard apolar stationary phase (Apolane-87) was not followed by the development of a standard polar stationary phase, although this was attempted. Although some important results have been achieved without using the polar counterpart, the special advantages offered by mixed stationary phases (*e.g.*, computer-assisted polarity design, resolution optimization by mixed stationary phases) could be exploited only if both polar and apolar stationary phases are applied together.

For detailed information, see refs. B10, 10, 85, 86, 176, 216, 218, 256, 306, 379, 379a and 465.

TABLE 86

CORRECTED RETENTION INDICES OF McREYNOLDS STANDARDS OBTAINED BY KERSTEN AND POOLE[354] ON DIFFERENT STATIONARY PHASES AT 80°C

Standard	*Retention index (i.u.)*			
	Squalane	*OV-225*	*PEG-20M*	*OV-275*[a]
Benzene	642	861	973	1104
n-Butanol	580	953	1151	1285
2-Pentanone	620	951	1000	1205
Nitropropane	642	1117	1232	1437
Pyridine	685	1052	1201	1397
2-Methyl-2-pentanol	682	979	1101	1217
Iodobutane	806	1017	1094	1172
2-Octyne	842	990	1074	1071
1,4-Dioxane	643	971	1089	1294
cis-Hydrindane	989	1090	1135	1116

[a] Uncorrected values.

TABLE 87

COMPARISON OF CORRECTED AND UNCORRECTED RETENTION INDICES OF McREYNOLDS STANDARDS OBTAINED BY KERSTEN AND POOLE[354] ON OV-225 AT 80°C

Standard	*Retention index (i.u.)*		
	Corrected	*Uncorrected*	*Difference*
Benzene	861	853	8
n-Butanol	953	953	0
2-Pentanone	951	947	4
Nitropropane	1117	1112	5
Pyridine	1052	1050	2
2-Methyl-2-pentanol	979	970	9
Iodobutane	1017	1013	4
2-Octyne	990	987	3
1,4-Dioxane	971	970	1
cis-Hydrindane	1090	1086	4

TABLE 88

RETENTION POLARITY OF SOME STATIONARY PHASES AT 120°C, ON THE BASIS OF McREYNOLDS CONSTANTS[439]

McReynolds code number	*Stationary phase*	*Retention polarity (polarity units)*
2000	Squalane	0.00
2226	Hexatriacontane	0.69
2063	Nujol	1.01
2298	Mineral oil	1.16
2270	Liquid paraffin	1.19
–	Apolane-87	2.30
2128	Apiezon M	4.24
2127	Apiezon L	4.39
2013	Apiezon B (treated)	4.49
2082	Polybutene-32	4.82
2081	Polybutene-128	4.90
2012	Apiezon L	5.11
2318	DC-330	6.37
2195	Apiezon L	6.38
2066	SE-96	6.43
2140	Apiezon N	6.67
2077	SE-30	6.80
2034	OV-1	6.95
–	OV-73	6.95
2050	M and B silicone oil	7.11
–	DC-200	7.11
–	SP-2100	7.17
–	ASI 100 methyl	7.17
2039	OV-101	7.17
2076	DC-410	7.33
2065	Versilube F-50	7.51

(*Continued on p. 56*)

TABLE 88 (*continued*)

McReynolds code number	*Stationary phase*	*Retention polarity (polarity units)*
2101	DC-11	8.69
2316	DC-510	9.24
2087	DC-200	10.18
2078	SE-52	10.42
2320	SE-54	10.51
–	SP-400	10.67
2314	DC-556	12.18
2204	OV-3	13.17
2100	Beeswax	13.18
–	OV-105	14.45
–	Dexil 300	14.72
2144	Fluorolube HG-1200	15.30
2194	Apiezon H	15.63
2080	Halocarbon wax	15.70
2171	Butoxyethyl stearate	15.89
2123	OV-7	18.39
2120	DC-550	19.25
2323	Apiezon W	19.41
2179	Dinonyl sebacate	19.90
2061	DC-703	20.32
2052	Dioctyl sebacate	20.39
2178	Di(2-ethylhexyl) sebacate	20.44
2056	Diisodecyl adipate	20.91
2169	Ditridecyl phthalate	21.46
2149	Diethoxy tetrachlorophthalate	21.47
2310	DEG stearate	21.74
2250	Dilauryl phthalate	22.01
2313	Octyl decyl adipate	22.04
–	SP-1200	22.08
2170	Diisooctyl adipate	22.20
–	Bis(2-ethylhexyl) tetrachlorophthalate	22.77
2024	TMP tripelargonate	22.77
2057	Diisooctyl adipate	23.01
2187	Diisodecyl phthalate	23.93
2124	OV-11	24.37
–	OV-1701	24.58
2070	Dinonyl phthalate	25.07
2229	DC-710	25.64
2060	Dioctyl phthalate	25.93
2276	Flexol GPE	25.94
–	Poly-I 110	26.03
2103	Hallcomid M-18	26.64
2116	Diisooctyl phthalate	26.77
2038	OV-17	27.40
–	SP-2250	27.40
2114	Hallcomid M-18 OL	28.83
2205	SP-392	29.60
2022	Flozol 8N8	29.88
2291	Span-60	30.17
2247	Hercoflex-600	30.28

TABLE 88 (*continued*)

McReynolds code number	*Stationary phase*	*Retention polarity (polarity units)*
2162	Versamid	30.46
2059	UCON LB-550-X	31.19
2069	Span-80	31.70
2049	UCON 50-HB-1800-X	31.93
2096	Castor wax	31.94
2254	Flexol B-400	32.88
2175	Estynox	33.29
2125	OV-22	33.29
2190	Trimer acid	33.66
–	Poly-A 103	33.69
2266	Atpet-200	33.92
2264	Pluracol P-2010	33.97
2113	UCON LB-1715	34.16
2182	Dibutoxyethyl adipate	34.28
–	Poly-A 101A	34.60
2253	Thanol PEG 1000	35.01
2044	OV-25	35.86
2160	Acetyl tributyl citrate	35.96
2007	Pluronic L-81	36.01
2177	Didecyl phthalate	36.14
2068	OS-124	36.37
2111	Tributyl citrate	37.01
2319	GE SR-119	37.71
2017	OS-138	38.06
2147	Diethoxyethyl sebacate	38.39
2048	Dibutoxyethyl phthalate	38.73
–	SP-1220	39.35
2174	Dibutoxyethyl phthalate	39.38
2108	NPG sebacate	39.43
2132	Squalane	40.60
–	Poly-A 135	41.66
2046	UCON 50-HB-280-X	43.41
2131	Polytergent J-300	43.74
2164	Paraplex G-25	44.27
2047	Tricresyl phosphate	44.34
2085	SAIB	44.34
2302	Ethomeen 18/25	44.50
2180	Polytergent J-400	44.75
2025	Oronite NIW	45.75
2086	QF-1	45.99
2252	UCON 50-HB-660	46.56
2093	PPG sebacate	46.63
2126	OV-210	46.78
2021	Ethofat 60/25	47.18
–	OV-202	47.18
–	SP-2401	47.18
2251	UCON 50-HB-3520	47.20
–	SP-2401	47.22
2062	Ethomeen S-125	47.53
2261	Igepal CO-630	47.77

(*Continued on p. 58*)

TABLE 88 (*continued*)

McReynolds code number	*Stationary phase*	*Retention polarity (polarity units)*
2092	LSX-3-0295	47.86
–	OV-215	47.95
2005	Pluronic P-65	48.32
2008	Pluronic P-85	48.43
2067	Tergitol NPX	48.74
2176	Emulphor ON-870	49.32
2183	Cresyl diphenyl phosphate	49.36
2003	Pluronic I-35	49.37
2184	Polytergent G-300	50.46
2228	Polyglycol 15-200	50.83
2122	Triton X-100	50.98
2129	Stepan DS-60	51.02
–	OV-330	52.08
2055	UCON 50-HB-5100	53.09
2146	Diethoxyethyl phthalate	53.15
2091	Siponate DS-10	53.24
2075	XE-60	53.83
2102	Renex-678	54.18
2045	OV-225	56.41
2185	Bis(ethoxyethoxyethyl phthalate	56.41
2138	NPGA	56.63
2300	UCON 75-H-90 000	57.57
2004	Pluronic F-88	58.70
2037	HI-EFF 8BP	60.22
2020	Igepal CO-880	60.45
2051	Triton X-305	61.14
2142	CW-4000 monostearate	62.40
2152	Zonyl E-7	63.97
2094	CW-4000 monostearate-2	64.79
2215	NPGS	65.23
2090	Quadrol	65.77
2098	Igepal CO-990	65.84
2042	EGSP-Z	67.51
2028	Carbowax(PEG)-20M	71.91
2084	Epon-1001	71.91
2029	Carbowax(PEG)-6000	71.95
2079	Ethylene glycol isophthalate	72.29
2315	Carbowax(PEG)-4000	73.32
–	OV-351	74.49
–	SP-1000	75.02
–	SP-2300	75.39
2153	XF-1150	75.43
–	Silar-5CP	75.51
2133	Sorbitol hexaacetate	77.68
2135	FFAP	78.86
2089	STAP	79.14
2026	Carbowax(PEG)-1000	79.84
2196	Sucrose octaacetate	80.59
–	PEG-1500	80.59
2334	MER-2	81.43

TABLE 88 (*continued*)

McReynolds code number	*Stationary phase*	*Retention polarity (polarity units)*
2119	PEG-600	81.90
2208	Butanediol succinate	82.46
2212	EGA	82.61
2139	Butanediol succinate-2	83.11
2219	PDEAS	84.19
2151	Reoplex-400	85.09
2157	LAC IR-296	85.50
2209	DEG adipate	85.61
2027	Carbowax(PEG)-1540	86.24
2304	Resoflex-R-296	86.63
2225	LAC-2-R-446	87.67
2041	EGSS-Y	88.11
2339	Hyprose SP-80	91.54
–	EGSP-A	92.37
–	SP-2310	99.31
–	Silar-7CP	99.55
–	ECNSS-S	99.62
2040	ECNSS-M	100.39
2338	Diglycerol	102.40
2107	DEGS Supelco-1045	102.55
2036	EGSS-X	105.30
2325	DEGS	106.63
2327	Ethylene glycol phthalate	106.69
2210	DEGS Supelco-1303	108.87
–	Silar-9CP	109.95
–	SP-2330	109.95
2303	DEGS-2	110.03
2224	LAC-3-R-728	110.43
–	SP-2340	114.39
–	Silar-10CP	114.51
2329	Glycol succinate	115.21
2110	THEED	116.20
2097	Tetracyanoethoxy PE	116.38
2213	EGS	116.77
–	SP-216-PS	122.53
–	1,2,3,4,5,6-Hexakis(cyclo-N)	123.86
2035	TCEP	128.91
–	OV-275	131.38
–	1,2,3,4-Tetrakis(cyclo-B)	131.78
2099	Cyanoethylsucrose	136.31
2117	BCEF	144.60

12. PRECALCULATION OF RETENTION INDEX

The widespread use of personal and home computers in the laboratory has given an impetus to such applications, but the progress is far from satisfactory. The problem is not connected with the computer software, but with the GLC itself. In spite of the fact that the theoretical background needed for the calculations is almost complete,

there is a dearth of reliable GC data for use in calculations, especially in those instances where the calculations are to be extended over different column temperatures.

We consider as examples papers of Calixto *et al.*[52], Szulc *et al.*[86], Bermejo and Guillen[102], Jánosi[118], Morishita *et al.*[206], Peetre *et al.*[211], Podmaniczky *et al.*[212], Betts[248], Mihara and Masuda[363], Fernandez-Sanchez *et al.*[379], Papazova *et al.*[421] and Peng *et al.*[422]. Some of their most interesting results are summarized in Tables 89–103.

From the many studies in this field we refer to those involving different mathematical aspects. Equations for retention indices *versus* Van der Waals volume on squalane stationary phase were studied by Calixto and Raso[8] for alcohols, carbonyls, esters and ethers. A method of calculating the specific retention volumes of compounds from their retention indices was presented by Golovnya and Grigoryeva[58a]. Relationships between GC retention indices of aromatic hydrocarbons and several parameters were demonstrated by Calixto and Raso[104a]. Calculation of retention indices of chlorinated alkanes was presented by Sabljić[141]. A new equation for the calculation of the retention indices of aliphatic saturated esters on any stationary phases was derived by Bermejo and Guillen[166].

Retention indices of C_6 epoxides on OV-101 stationary phase at four column temperatures between 60 and 90°C were determined by Boneva and Dimov[253]. Linear regression equations permit the precalculation of the retention indices. Application of a three-factor model on 24 stationary phases was reported by Howery *et al.*[280]. Retention indices of 42 solutes showed good agreement with predictions. Precalculation of the retention indices of isoalkanes on squalane was proposed by Dimov[335]. Prediction of retention data using the slope of the log(adjusted retention time) *versus* carbon number plot for *n*-alkanes was discussed by Hawkes[413].

For further information, see also refs. 26, 40, 49, 50, 72, 74, 78, 102, 105a, 116, 138, 163, 167, 170a, 183, 206, 207, 267a, 278, 288, 291, 293, 303, 313, 323, 324, 343, 348, 357, 383, 391, 407, 422, 467 and 475.

TABLE 89

COMPARISON OF RETENTION INDICES OF SOME ALCOHOLS MEASURED AND CALCULATED BY CALIXTO *ET AL.*[52] ON PEG-1540 AT 90°C

Alcohol	*Retention index (i.u.)*					
	Measured	*Calculation by equation in ref. 52*				
		1	*2*	*3*	*4*	*5*
Propanol	1039.3	−2.2	2.6	−2.2	−2.2	−2.2
n-Butanol	1149.6	0.9	3.2	0.9	0.9	0.9
n-Pentanol	1258.1	2.2	0.4	2.2	2.2	2.2
n-Hexanol	1363.1	0	−9.3	0	0	0
n-Heptanol	1470.4	0.1	−1.7	0.1	0.1	0.1
n-Octanol	1577.2	−0.3	3.1	−0.3	−0.3	−0.3
n-Nonanol	1684.1	−0.6	4.2	−0.6	−0.6	−0.7

TABLE 90

COMPARISON OF RETENTION INDICES MEASURED AND CALCULATED BY SZULC *ET AL.*[86] ON MIXED LIQUID-CRYSTALLINE STATIONARY PHASES AT 120°C

Symbol[a] of stationary phase	*Compound[b]*	*Retention index (i.u.)*		
		Measured	*Calculated*	*Difference*
$A_1 + B_2$	*o*-E_2B	1068.0	1059.4	8.6
	m-E_2B	1148.7	1142.1	6.6
	p-E_2B	1195.5	1188.9	6.6
$B_2 + C_1$	*o*-E_2B	1203.5	1195.8	7.7
	m-E_2B	1183.3	1175.9	7.4
	p-E_2B	1233.7	1224.2	9.5
$A_1 + C_1$	*o*-E_2B	1171.4	1170.4	1.0
	m-E_2B	1153.4	1152.1	1.3
	p-E_2B	1199.6	1199.7	0.1
$A_1 + B_2 + C_1$	*o*-E_2B	1171.3	1160.1	11.2
	m-E_2B	1151.9	1143.3	8.6
	p-E_2B	1198.9	1190.6	8.3
$A_1 + C_3$	*o*-E_2B	1164.9	1165.2	0.3
	m-E_2B	1145.6	1146.9	1.3
	p-E_2B	1188.4	1191.8	3.4
$C_1 + C_3$	*o*-E_2B	1204.7	1202.6	2.1
	m-E_2B	1181.9	1181.0	0.9
	p-E_2B	1224.9	1226.2	1.3
$A_1 + C_1 + C_3$	*o*-E_2B	1168.4	1164.8	3.6
	m-E_2B	1149.0	1147.0	2.0
	p-E_2B	1192.5	1192.5	0.0
$B_2 + C_3$	*o*-E_2B	1194.7	1190.5	4.2
	m-E_2B	1171.8	1169.8	2.0
	p-E_2B	1215.7	1215.1	0.6
$A_1 + B_2 + C_3$	*o*-E_2B	1163.9	1150.8	13.1
	m-E_2B	1144.4	1134.1	10.3
	p-E_2B	1187.2	1180.1	7.1

[a] Symbols as in ref. 85.

[b] E_2B = diethylbenzene.

13. RETENTION INDEX AND COMPUTERS (GC–MS)

Although the use of computers is becoming increasingly popular and many researchers have applied them in chromatogram evaluation (GC–MS), the possibilities offered have not been fully exploited.

A computerized rapid analysis system and its application to two research problems were described by Demirgian[106]. From the results published in this field we

TABLE 91

COMPARISON OF RETENTION INDICES MEASURED AND CALCULATED BY BERMEJO AND GUILLÉN[102] ON SQUALANE AT 50°C

Compound[a]	*Retention index (i.u.)*				
	Measured	*Calculated*		*Difference*	
		Method 1	*Method 2*	*Method 1*	*Method 2*
2-MC_4	474.9	468.4	469.1	6.5	5.8
2,2-M_2C_4	536.6	527.7	528.2	8.9	8.4
2,3-M_2C_4	567.6	562.5	562.5	5.1	5.1
2-MC_5	569.5	571.1	571.1	−1.6	−1.6
3-MC_5	584.0	584.4	584.1	−0.4	−0.1
2,2-M_2C_5	625.9	626.6	626.0	−0.7	−0.1
2,4-M_2C_5	629.9	631.7	631.3	−1.8	−1.4
2,2,3-M_3C_4	639.8	634.6	634.1	5.2	5.7
3,3-M_2C_5	658.9	656.1	655.4	2.8	3.5

[a] M = methyl; C_4 = butane; C_5 = pentane.

can mention by way of illustration those of Brooks *et al.*[46], Burns and Tingey[48], Marriott *et al.*[129], Maurer and Pfleger[130,131], Tandon *et al.*[148], Tong *et al.*[152], Gill and co-workers[175,267], Wu[316], D'Agostino *et al.*[318], Harvey *et al.*[349], Sakamoto *et al.*[376], Hajšlová *et al.*[412], Köppel *et al.*[416] and Matisová[418].

For further information, see also refs. 102a, 225, 289, 304, 402 and 479.

14. RETENTION INDEX LIBRARY

Similarly to retention index review papers, attempts to organize a retention index library[30,143,396,429a] have been only of a recapitulatory type, *i.e.*, they contain very few critical elements, if any. Thus if may often happen that different retention index values are given for the same compound measured with a particular GC system. As

TABLE 92

COMPARISON OF RETENTION INDICES OF 2-METHYLNAPHTHALENE MEASURED AND CALCULATED BY JÁNOSI[118] ON DIFFERENT OV STATIONARY PHASES AT 150°C

Stationary phase	*Retention index (i.u.)*		
	Measured	*Calculated*	*Difference*
OV-1	1294.0	1293.7	0.3
OV-3	1335.0	1335.0	0
OV-7	1373.0	1374.5	−1.5
OV-11	1433.0	1431.0	2.0
OV-17	1484.0	1484.7	−0.7
OV-22	1536.0	1536.6	−0.6
OV-25	1571.0	1570.6	0.4

TABLE 93

COMPARISON OF RETENTION INDICES MEASURED AND CALCULATED BY BERMEJO AND GUILLÉN[166] OF ALIPHATIC SATURATED ESTERS ON DIFFERENT STATIONARY PHASES AT 120°C

Compound	*Retention index (i.u.)*					
	Diisodecylphthalate		*Pluronic P65*		*Bis(2-ethoxyethyl)-phthalate*	
	Measured	*Calc.*	*Measured*	*Calc.*	*Measured*	*Calc.*
Methyl formate	482	+14	627	+10	641	+37
Ethyl acetate	659	+3	772	−2	801	0
Propyl formate	684	+4	802	0	828	+7
Propyl acetate	757	+7	867	0	897	0
Isopropyl propionate	780	+9	868	+14	905	+4
Isobutyl acetate	817	+8	914	+8	948	+2
Propyl propionate	846	+5	947	+3	980	0
Pentyl formate	892	−7	1010	−5	1043	−7
Isobutyl propionate	909	−1	999	+2	1036	−7
2-Ethyl 1-butylacetate	1025	0	1127	−2	1164	−10

TABLE 94

COMPARISON OF RETENTION INDICES MEASURED BY MORISHITA *ET AL.*[206] AND CALCULATED ON PEG-20M STATIONARY PHASE AT 93°C

Compound[a]	*Retention index (i.u.)*		
	Measured	*Calculated*	*Difference*
1-C_5SH	1052.9	1054.4	−1.5
1-C_7SH	1253.8	1252.6	+1.2
2-C_5SH	967.9	969.2	−1.3
2-C_7SH	1165.5	1164.6	+0.9
3-C_5SH	979.5	976.0	+3.5
3-C_6SH	1062.5	1061.5	+1.0
3-C_7SH	1157.6	1158.6	−1.0
4-C_7SH	1144.1	1146.7	−2.6
C_1-SC_4	1032.6	1036.2	−3.6
C_1-SC_5	1132.3	1133.3	−1.0
C_1-SC_6	1232.1	1231.6	+0.5
C_1-SC_7	1332.1	1331.4	+0.7
C_1-SC_8	1431.7	1431.5	+0.2
C_2-SC_4	1096.1	1095.9	+0.2
C_2-SC_5	1193.0	–	–
C_2-SC_6	1291.2	1291.3	−0.1
C_2-SC_7	1390.7	1391.1	−0.4

[a] C_1 = Methyl; C_2 = ethyl; C_4 = *n*-butyl; C_5 = *n*-pentyl; C_6 = *n*-hexyl; C_7 = *n*-heptyl; C_8 = *n*-octyl.

TABLE 95

COMPARISON OF RETENTION INDICES OF ALKYLALKOXYSILANES MEASURED AND CALCULATED BY PEETRE *ET AL.*[211] ON APIEZON M AT 160°C

Compound	*Retention index (i.u.)*		
	Measured	*Calculated*	*Difference*
$CH_3Si(OCH_3)_3$	624	624	0
$CH_3Si(OC_4H_9)_3$	1271	1272	−1
$CH_3Si(OC_5H_{11})_3$	1538	1539	−1
$CH_3Si(OC_6H_{13})_3$	1808	1811	−3
$CH_3Si(OC_7H_{15})_3$	2085	2083	2
$(CH_3)_2Si(OC_2H_5)_2$	678	677	1
$(CH_3)_2Si(OC_3H_7)_2$	847	846	1
$(CH_3)_2Si(OC_4H_9)_2$	1024	1022	2

TABLE 96

COMPARISON OF RETENTION INDICES MEASURED AND CALCULATED BY PODMANICZKY *ET AL.*[212] ON PEG-20M AT DIFFERENT COLUMN TEMPERATURES

Compound	*Retention index (i.u.)*					
	80°C		*100°C*		*120°C*	
	Measured	*Δ*[a]	*Measured*	*Δ*[a]	*Measured*	*Δ*[a]
Benzene	960.7	−0.2	968.7	−0.7	976.9	+2.0
Toluene	1058.0	−0.5	1067.0	−1.1	1076.1	−0.8
p-Xylene	1149.9	−0.1	1159.9	−1.2	1169.8	−1.6
m-Xylene	1156.3	+0.7	1166.4	−0.1	1176.3	0.0
o-Xylene	1198.3	+1.2	1209.8	−0.6	1221.1	−1.4

[a] Difference between measured and calculated values.

TABLE 97

COMPARISON OF RETENTION INDICES MEASURED AND CALCULATED BY BETTS[248] ON DIFFERENT STATIONARY PHASES AT 120°C

Stationary phase	*Retention index (i.u.)*					
	n-Butanol		*Pyridine*		*2-Octyne*	
	Measured	*Δ*[a]	*Measured*	*Δ*[a]	*Measured*	*Δ*[a]
Squalane	590	+20	699	+6	841	+11
OV-1	645	+18	741	−4	864	−5
SE-30	643	+2	740	+3	863	+3
SP-2100	647	+4	742	+4	866	−6
OV-17	748	−1	901	−30	946	−16
SP-2550	748	−10	901	−14	946	−10
OV-210	828	−13	1009	+32	897	+6
OV-225	959	+20	1085	+39	991	+11
SP-2330	1315	+52	1477	+80	1153	+56
PEG 1000	1197	+38	1288	+60	1081	+29
PEG 20M	1126	+22	1209	+29	1062	+21
DEGS	1323	+94	1490	+127	1162	+47

[a] Difference between measured and calculated values.

TABLE 98

COMPARISON OF RETENTION INDICES OF SOME ALKYLPYRIDINES MEASURED AND CALCULATED BY MORISHITA *ET AL.*[291] ON OV-101 AND PEG-20M AT 150°C

Compound	*Retention index (i.u.)*			
	OV-101		*PEG-20M*	
	Measured	Δ^a	*Measured*	Δ^a
Pyridine	744.6	+3.4	1241.5	+1.7
2-Methylpyridine	814.0	−2.1	1268.3	−3.2
3-Methylpyridine	859.1	+0.8	1354.3	+0.6
4-Methylpyridine	862.7	−1.6	1363.5	+1.0
2,3-Dimethylpyridine	940.3	+9.2	1410.5	+13.8
2,5-Dimethylpyridine	925.9	−4.0	1377.6	−6.2
2,6-Dimethylpyridine	875.8	−2.1	1287.9	+0.2
3,4-Dimethylpyridine	999.9	−1.2	1528.3	−4.9
3,5-Dimethylpyridine	972.4	−2.7	1468.7	−1.6
2,4,6-Trimethylpyridine	982.4	+0.2	1409.1	+1.3
2,4-Dimethylpyridine	923.9	−1.0	1390.1	−2.9

[a] Difference between measured and calculated values.

TABLE 99

COMPARISON OF RETENTION INDICES MEASURED AND CALCULATED BY MIHARA AND MASUDA[363] ON OV-101 AND PEG-20M AT 70°C

Compound	*Retention index (i.u.)*			
	OV-101		*PEG-20M*	
	Measured	*Calculated*	*Measured*	*Calculated*
Methylpyrazine	801	800	1235	1235
2,3-Dimethylpyrazine	897	894	1309	1307
2,5-Dimethylpyrazine	889	890	1290	1293
2,6-Dimethylpyrazine	889	887	1300	1296
Trimethylpyrazine	981	981	1365	1367
Tetramethylpyrazine	1067	1072	1439	1441
Ethylpyrazine	894	891	1300	1300
2-Ethyl-5-methylpyrazine	980	981	1357	1357
2-Ethyl-6-methylpyrazine	977	973	1353	1351
2,5-Dimethyl-3-ethylpyrazine	1059	1059	1400	1403
2,6-Dimethyl-3-ethylpyrazine	1064	1064	1415	1412
2,3-Dimethyl-5-ethylpyrazine	1066	1067	1421	1422
2,3-Diethylpyrazine	1065	1060	1417	1413

TABLE 100

COMPARISON OF RETENTION INDICES MEASURED AND CALCULATED BY FERNÁNDEZ-SANCHEZ *ET AL.*[379] ON DIFFERENT MIXED STATIONARY PHASES AT 120°C

McReynolds standard	*Retention index (i.u.)*								
	OV-225 + SP-2340 (78.1:21.9)			*OV-225 + SP-2340 (51:49)*			*OV-225 + SP-2340 (10.3:89.7)*		
	Measured	*Calculated*	*Difference*	*Measured*	*Calculated*	*Difference*	*Measured*	*Calculated*	*Difference*
Benzene	893	893	0	953	958	−5	1125	1124	+1
1-Butanol	988	991	−3	1069	1073	−4	1290	1286	+4
2-Pentanone	981	983	−2	1044	1042	+2	1197	1198	−1
1-Nitropropane	1171	1173	−2	1259	1265	−6	1515	1503	+12
Pyridine	1114	1114	0	1198	1200	−2	1438	1430	+8
2-Methyl-2-pentanol	988	989	−1	1045	1051	−6	1233	1227	+6
1-Iodobutane	1047	1047	0	1088	1091	−3	1237	1233	+4
2-Octyne	997	995	+2	1013	1020	−7	1108	1106	+2
1,4-Dioxane	1021	1023	−2	1098	1100	−2	1320	1314	+6
cis-Hydrindane	1118	1119	−1	1141	1145	−4	1226	1222	+4

TABLE 101

COMPARISON OF MEASURED AND CALCULATED RETENTION INDICES ON SQUALANE AT 30°C, AFTER PAPAZOVA *ET AL.*[421]

Substance	*Retention index (i.u.)*		
	Measured	*Calculated*	*Difference*
cis-3-Hexene	592.3	591.5	+0.8
2-Ethyl-1-butene	592.0	590.5	+1.5
trans-2-Hexene	597.3	596.6	+0.7
trans-3-Hexene	592.8	594.2	+1.4
cis-2-Hexene	603.2	601.5	+1.7
3,3-Dimethyl-1-pentene	624.1	623.7	+0.4

TABLE 102

COMPARISON OF MEASURED (BY TPGC) AND CALCULATED RETENTION INDICES OF AROMATIC HYDROCARBONS ON SE-30, AFTER PENG *ET AL.*[422]

Compound	*Retention index (i.u.)*		
	Measured	*Calculated*	*Difference*
Benzene	654	658	−4
Toluene	764	768	−4
Ethylbenzene	858	858	0
o-Xylene	895	893	+2
m-Xylene	871	878	−7
p-Xylene	875	878	−3
n-Butylbenzene	1058	1058	0
Biphenyl	1375	1376	−1
Octahydroanthracene	1684	1654	+30
p-Terphenyl	2121	2094	+27

TABLE 103

COMPARISON OF MEASURED (BY TPGC) AND CALCULATED RETENTION INDICES OF ALCOHOLS ON SE-30, AFTER PENG *ET AL.*[422]

Compound	*Retention index (i.u.)*		
	Measured	*Calculated*	*Difference*
2-Methyl-1-propanol	626	626	0
3-Methyl-1-butanol	726	716	+10
3-Methoxy-1-butanol	814	816	−2
2-Ethyl-1-butanol	836	816	+20

TABLE 104

OUTLINE OF A PAGE IN A PROPOSED INTERNATIONAL RETENTION INDEX LIBRARY[a]

Substance: Benzene

Retention data:

I. 1 Stationary phase: OV-101.

2. Temperature range of appicability: 0–350°C.

3 Kováts coefficients at different frequently used column temperatures:

Column temperature (°C)	*Kováts coefficient [dim.][b]*
0	53.24
10	61.10
30	78.17
50	97.29
70	118.77
90	143.03
100	156.22
110	170.34
120	185.43
130	201.44
150	237.84

4. *b* Values at different frequently used column temperatures:

Column temperature (°C)	*b Value*
0	0.5171
10	0.4868
30	0.4323
50	0.3847
70	0.3427
90	0.3055
100	0.2884
110	0.2722
120	0.2568
130	0.2422
150	0.2152

5. Retention indices at different frequlently used column temperatures:

Column temperature (°C)	*Retention index (i.u.)*
0	631.9
10	635.3
30	642.0
50	648.3
70	654.3
90	660.0
100	662.8
110	665.5
120	668.1
130	670.7
150	678.2

TABLE 104 (*continued*)

II. 1. Stationary phase: Apolane-87.
2. Temperature range of applicability: 30–280°C.
3. Kováts coefficients at different frequently used column temperatures:

Column temperature (°C)	*Kováts coefficient [dim.][b]*
30	73.71
40	80.39
50	86.92
70	99.52
90	111.54
100	117.34
110	123.02
120	128.57
130	134.00
150	144.51

4. *b* Values at different frequently used column temperatures:

Column temperature (°C)	*b Value*
30	0.4299
40	0.4090
50	0.3894
70	0.3536
90	0.3217
100	0.3071
110	0.2932
120	0.2800
130	0.2675
150	0.2442

5. Retention indices at different frequently used column temperatures:

Column temperature (°C)	*Retention index (i.u.)*
30	646.1
40	649.4
50	652.7
70	659.2
90	665.7
100	669.0
110	672.3
120	675.5
130	678.8
150	685.3

III. 1. Stationary phase: Squalane.
2. Temperature range of applicability: 0–125°C.
etc.

[a] The International Retention Index Library is sponsored.

[b] Carrier gas: helium.

a consequence, the user has to decide which value to take as the proper one. In other instances the published data and/or retention index library are reliable, but incomplete to a large extent.

The best solution would be the establishment of an international *ad hoc* committee sponsored by those companies which produce and sell stationary phases, supports, and open-tubular (capillary) columns. Moreover, it would be advantageous to obtain additional support from companies manufacturing gas chromatographs and integrators and/or computers for GLC.

Prior to the beginning of the extensive collection of data, this *ad hoc* committee should undertake the selection of those relationships which represent the theoretical basis (retention index calculations, measurement and calculation of the dead time, etc.), then make them available to those who participated in the measurement and collection of data.

Table 104 outlines a data sheet in a future International Retention Index Library.

For further information, see ref. 215.

15. LIST OF SYMBOLS USED

α	relative volatility;
a_0	atomic code number in the bond;
A	constant in eqn. 1;
a^R	substance-specific factor according to Rohrschneider;
a_1	atomic code number in the primary environment;
a_2	atomic code number in the secondary environment;
B	constant in eqn. 1;
b^R	substance-specific factor according to Rohrschneider;
b	slope of *n*-alkane plot;
C	constant in eqn. 1;
c^R	substance-specific factor according to Rohrschneider;
d	bond length;
d^R	substance-specific factor according to Rohrschneider;
D	constant in eqn. 9; dimension of S_c and/or K_c; [dim.] = log [cm^3 (carrier gas)/g (stationary phase)];
E	constant in eqn. 9;
e^R	substance-specific factor according to Rohrschneider;
E_{C-H}	bond energy of alkane C–H bond;
E_{C-C}	bond energy of alkane C–C bond;
f_c	index units/energy units conversion factor for alkanes;
f_s	[dim.]/energy units conversion factor for alkylbenzenes;
ΔG	partial molar free energy of solution;
I	isothermal retention index;
i.u.	retention index units;
i	serial number;
i_b	bond index increment;
i_i	interaction index increment;
i_s	structural increment;
ΔI	$I_s^{st.ph.}(T) - I_s^{SQ}(T)$;
j	serial number;

k serial number;
K_c Kováts coefficient, $\{-\log [cm^3$ (carrier gas)/g (stationary phase)]$\}$;
n-C_z n-alkane with carbon number z;
p total number of atoms of the bond in the primary environment;
p.u. polarity units;
q total number of atoms of the bond in the secondary environment;
s substance of interest;
s_c molecular structural coefficient, $\{\log [cm^3$ (carrier gas)/g (stationary phase)]$\}$;
st.ph. stationary phase;
SQ squalane stationary phase;
s_i ith substance-specific factor;
t_M gas holdup (dead) time;
t'_R adjusted retention time;
t_N net retention time;
T the column-temperature;
TPGC or TP temperature-programmed gas chromatography;
V_g specific retention volume;
V_N net retention volume;
X retention value used in calculation;
z carbon number of n-alkane.

16. LIST OF COMPOUNDS EXAMINED

To facilitate the use of this review, retention indices are grouped in Table 105 based on the type of chemical compound.

TABLE 105

REFERENCES TO RETENTION INDICES OF DIFFERENT COMPOUNDS

Compound type	*References*
Acids	422
Adamantanes	246
Alcohols	8, 19, 58a, 120, 281, 284, 324, 329, 343, 349, 422
Aldehydes	93
Aldoses	366
Alicyclic hydrocarbons	340
Aliphatic acids	281
Alkaloids	260
Alkanes	2, 7, 19, 35b, 36, 48, 54, 55, 75, 93a, 161, 173, 250, 258, 309, 325, 333, 335, 407, 413, 422, 428, 430
Alkanols	5
Alkenes	7, 17, 19, 54, 55, 93a, 138, 155, 173, 249, 258, 297, 316, 422
Alkenylbenzenes	160, 339
Alkyl acrylates	182
Alkylalkoxysilanes	211
Alkyl chlorides	257
Alkyldecenes	208
Alkylnaphthalenes	118
Alkylphenols	409
Alkylporphyrins	129, 175, 267
Alkylquinolines	332

(*Continued on p. 72*)

TABLE 105 (*continued*)

Compound type	*References*
Alkylserines	376
Alkynes	208
Amidines	292, 295, 296
Amines	145, 209, 225a, 403, 422
Amino ether alcohols	384, 388
Amino acids	219
Anthracenes	287
Anthraquinones	201
Antidepressants	130
Aromatics	7, 19, 42, 44, 54, 55, 58a, 101, 104a, 170a, 258, 285, 286, 309, 418, 422, 424a, 429a, 433
Arylaliphatic acids	198
Azaarenes	291
Barbituric acids	25
Benzodiazepines	414
Benzophenones	414
Benzothiophenes	243, 415
Benzylideneamines	210
Beta-adrenolytics	417
Beta-blockers	289
Bicyclo-2-alkenones	212b
Carbohydrates	374
Carbonyls	8
Chlorinated alkanes	78, 141
Chlorinated anisoles	123
Chlorinated benzenes	62, 167, 185, 214, 247
Chloroallyl compounds	137
Chloroanisoles	186
Chloroethanols	191
Chlorophenols	125, 192
Chlorovinyl compounds	137
Chlorinated dibenzo-*p*-dioxins	337
Chlorinated hydrocarbons	71
Chlorinated 4-hydroxybenzaldehydes	122
Chlorinated salicylaldehydes	124
Chlorinated veratroles	121
Cumene	23
Cyclic ferroceneboronates	331
Cyclic hydrocarbons	7, 19, 54, 55, 286
Cycloalkanes	102
Cyclohexenes	27, 103
Cycloolefins	327
Cyclopentadienes	381
Dibenzothiepins	232
Dichlorobenzyl alkyl ethers	238
Dienes	422
Dimethylbenzamidines	210
Dioxides	243
Diterpenes	136
Drugs	40a, 255, 364, 396
Esters	8, 58a, 60, 61, 63, 65, 93, 111–113, 119, 166, 177–179, 180, 188–190, 193, 194–196, 269, 270, 275, 276, 281–284, 343, 359, 388, 422

TABLE 105 (*continued*)

Compound type	*References*
Epoxides	253, 330
Essential oils	114, 115
Ethers	8, 58a, 228, 343, 384, 422
Fatty acids	349
Fusariotoxins	41
Glycidic esters	300
Halides	182
Halocarbons	10, 19a
Halogenated alkanes	404
Halogenated anisoles	271, 341
Halogenated benzenes	271, 341
Halogenated cyclohexanes	271, 341
Halogenated olefins	404
Halogenated phenols	422
Halogenocarboxylic acids	236
Halopropionates	281
Heterocyclics	422
Hydrazones	144
Hydroxy acids	102a
Irritants	158
Ketones	58a, 93, 213, 343, 422
Ketonic bile acids	148
Lemon oil	256
Monocyclic terpene alcohols	159
Mycotoxins	1
Naphthalenes	7, 118
Neuroleptics	131, 170
Nitrated polyaromatic hydrocarbons	92, 187, 301–303
Non-ionic surfactants	310, 387
Olefins	127
Organic acids	37a
Organophosphorus compounds	158
Perfluorocarbons	79
Permethylated oligosaccharide alditols	91
Petroleum fractions	87, 383, 421
Phenols	39a, 422
Phenoxypropanoic acid derivatives	412
Phenylacetic acid derivatives	204
Phenylpropionic acid derivatives	204
Polyaromatic hydrocarbons	38, 72, 303
Polychlorinated biphenyls	197
Poly(ether alcohols)	109
Pristane and other isoprenoids	6
Pyrazines	288, 363
Pyridines	372
p-Quinones	361
Sedative hypnotic drugs	134
Simulants	158
Steroidal alkaloids	410
Steroids	46, 95
Sterols	45

(*Continued on p. 74*)

TABLE 105 (*continued*)

Compound type	*References*
Substituted salicylic acids	402
Sulphides	206
Sulphur compounds	228
Sulphur(II)-containing compounds	174a
Sulphur vesicants	398
Thianes	415
Thiols	94, 206
Thiophenes	415
Toxic compounds	151
Trichothecene toxins	97, 147
Vesicants	158

17. LIST OF STATIONARY PHASES

Retention indices measured on different stationary phases are listed in Table 106.

TABLE 106

REFERENCES TO RETENTION INDICES MEASURED ON DIFFERENT STATIONARY PHASES

Stationary phase	*References*
Acetyltributyl citrate	23, 42, 285
Apiezon K	387
Apiezon L	19, 19a, 58, 78, 88, 105, 141, 246, 257
Apiezon M	58, 167, 211
Apolane-87	53, 198, 271, 341
BP-1	327
Cation exchanger	17, 96, 183
Cyanopropylsilicone	4, 406
CHOB	35b
Citroflex-A4	418
Carbowax: see PEG	
DB-1	45, 48, 75, 91, 127, 134, 135, 158, 175, 197, 201, 318, 398, 418
DB-5	45, 75, 127, 134, 152, 158, 243, 304, 318, 372, 398
DB-1701	318
DC-550	206
Dexsil-300	36, 232, 376
Dexsil-400	232
Dexsil-410	232
Di-2-ethylhexyl sebacate	2
Dioctyl phthalate	166, 296, 324, 375
DEGS	49, 50, 79, 163, 248, 355, 361, 374
Emulphor-ON-870	19
FFAP	125, 185
Fluorinated	369
HP-1	319
Hallcomid M-18	149
JXR	421
Liquid alkylammonium sulphonates	345, 368, 423, 424a
Liquid crystalline	85, 86, 117, 286

TABLE 106 (*continued*)

Stationary phase	*References*
Mixed	71, 86, 176, 218, 306, 337, 379
Methylpolysiloxane	421
N-Lauroyl-(*R*)-α-(1-naphthyl)ethylamine	236
NPP	35b
OBO	35b
Oronite NIW	166, 324
OV-101	25, 44, 54, 72, 73, 93a, 111, 130, 131, 144, 167, 169, 170, 182, 200, 204, 209, 219, 249, 253, 255, 258, 288, 289, 291, 297, 306, 327, 329, 330, 332, 363, 367, 373, 379, 379a, 392, 394, 413, 418
OV-1	36, 37a, 46, 58a, 84, 118, 129, 137, 160, 163, 248, 331, 339, 340, 404, 417
OV-3	55, 118, 258, 413
OV-7	55, 72, 118, 258, 413
OV-11	55, 118, 258, 413
OV-17	37a, 55, 72, 73, 118, 144, 163, 169, 170, 200, 204, 248, 258, 355, 361, 374, 376, 379a, 387, 417, 430
OV-22	55, 118, 258
OV-25	55, 65, 118, 123, 124, 163, 258, 306, 359, 379a, 413
OV-61	413
OV-105	375
OV-210	167, 248, 327
OV-215	4, 93, 327, 373
OV-225	25, 76, 77, 144, 159, 163, 248, 295, 354, 355, 366, 367, 373, 379
OV-275	79, 354, 355
OV-351	60, 61, 63, 119–121, 177–180, 185, 186, 188–196, 269, 270, 282–284
OV-1701	158, 327, 398, 409
PEG-400	23, 285
PEG-1000	58, 65, 79, 166, 248, 324
PEG-1540	2, 23, 51, 52, 104, 218, 285, 343
PEG-4000	27
PEG-20M	5, 6, 23, 27, 42, 49, 50, 58, 62, 78, 87, 88, 101, 103, 105, 112–115, 136, 137, 149, 159, 161, 167, 185, 206, 208, 212, 212a, 214, 243, 248, 256, 257, 271, 285, 288, 291, 297, 298, 329, 332, 340, 354, 355, 361, 374, 379, 381, 392
Pluronic-F88	166, 324
Porapak S, PS, Q, R	90
PPG-425	323
QF-1	65, 79, 355, 387
SE-30	4, 7, 23, 36, 40a, 58, 58a, 60, 61–63, 65, 72, 87, 90, 93, 95, 103, 109, 112, 113, 119–125, 136, 148, 149, 151, 161, 166, 177–180, 186–196, 210, 213, 214, 260, 269, 270, 282–284, 285, 292, 295, 296, 324, 359, 361, 364, 374, 387, 414, 418
SE-52	1, 4, 12, 41, 72, 92, 97, 147
SE-54	46, 182, 241, 247, 256, 366, 374
Sil-5	238, 298, 410
Silar-10C	4, 65, 76, 77
Siponate DS-10	93
SP-255	409

(*Continued on p. 76*)

TABLE 106 (*continued*)

Stationary phase	*References*
SP-400	281
SP-1000	10, 182, 212, 212a, 404
SP-2100	54, 227, 248, 298
SP-2250	248
SP-2330	248
SP-2340	379
Siloxane/silarylene	322
Squalane	2, 8, 23, 49, 50, 64, 79, 90, 101, 102, 104, 138, 155, 173, 218, 246, 247, 285, 297, 316, 327, 339, 343, 354, 355, 358, 365, 382, 385, 418, 421
TCEP	23, 42, 163, 285, 355
Tetrakis(2-cyanoethoxy)butane	271, 341
Tricresyl phosphate	257
Triton X-305	58, 65
TXP	167, 247
UCON 50-HB-280X	42
UCON 50-HB-250	285
UCON LB-550X	2, 23, 42, 167, 182, 247, 285, 300, 332, 340, 361, 418
XE-60	211, 361
Wax-51	93, 350

18. LIST OF IMPORTANT RETENTION INDEX RESEARCH ESTABLISHMENTS

To help with communication between researchers in this field, the addresses of some important retention index establishments are presented in Table 107.

TABLE 107

LIST OF IMPORTANT RETENTION INDEX RESEARCH ESTABLISHMENT

Country	*Establishment*
Algeria	Institute de Chimie, Laboratoire de Chromatographie, B.P. 32, El-Alia, Bab-Ezzour, Algiers
Saudia Arabia	University of Petroleum and Minerals, Dhahran
Australia	Department of Polymer Science, University of New South Wales, P.O. Box 1, Kensington, N.S.W., Australia 2033
Belgium	Service de Chimie Générale et Chabochimie, Faculté des Sciences Appliquées (CP 165), Université Libre de Bruxelles, Avenue Franklin Roosevelt 50, B-1050 Bruxelles
	Research Institute for Chromatography, P.O.B. 91, B-8610 Wevelgem
	Laboratory of Organic Chemistry, University of Ghent, Kriigshan 281(S4), B-9000 Gent
Bulgaria	Institute of Chemical Technology, 8010 Burgas
	Chemical Pharmaceutical Institute, 1156 Sofia
	University of Sofia, Faculty of Chemistry, Bulv. Anton Ivanov 1, 1126 Sofia
	Petrochemical Research Institute, Economic Combine "Neftochim", 8104 Burgas

TABLE 107 (*continued*)

Country	*Establishment*
Canada	Defence Research Establishment Suffield, Ralston, Alberta TOJ 2NO
	Science and Technology Advisory Group, "L" Directorate, R.C.M. Police, Ottawa, Ontario
China	Department of Chemistry, Peking University, Beijing
	Beijing Institute of Labour Hygiene and Occupational Diseases, Dong Da Qiao, Beijing
Czechoslovakia	Research Institute for Pharmacy and Biochemistry, 130 60 Prague 3
	Pharmaceutical Faculty, Charles University, Hradec Kralové
	Research Institute for Rheumatology, Na Slupi 4, 128 50 Prague
	Department of Analytical Chemistry, Charles University, Hlavova 2030, Prague
	Chemical Institute, Comenius University, 842 15 Bratislava
	Department of Analytical Chemistry, Institute of Chemistry, Institute of Chemical Technology, 532 10 Pardubice
	Institute of Chemical Technology, Suchbatarova 5, Prague 6
	Research Institute for Cruide Oil and Hydrocarbon Gases, Vlcie Hrdlo, 824 17 Bratislava
	Institute of Analytical Chemistry, Chechoslovak Academy of Sciences, Leninova 82, 611 42 Brno
	Institute of Experimental Pharmacology, Centre of Physiological Sciences, Slovak Academy of Sciences, 842 16 Bratislava
	Department of Analytical Chemistry, Faculty of Chemical Technology, Slovak Technical University, 812 37 Bratislava
F.R.G.	Bundesanstalt für Materialprüfung, Unter den Eichen 87, D-1000 Berlin 45
	Abteilung Analytische Chemie, Universität Ulm, D-7900 Ulm
	Laboratorium für Organische Chemie der Universität Bayreuth, Postfach 3008, D-8580 Bayreuth
	Medizinische Klinik und Poliklinik der Universität Göttingen, Robert Koch Strasse 40, D-3400 Göttingen
	Institute of Forensic Medicine, Karl-Ruprecht-University, Vossstr. 2, D-6900 Heidelberg
	Max-Planck-Institut für Kohlenforschung, Kaiser-Wilhelm-Platz 1, Mülheim/Ruhr
Finland	Department of Chemistry, University of Jyväskylä, Kyllikinkatu 1–3, SF-40100 Jyväskylä 10
France	Université de Recherche Scientifique, Avenue Phillipon, 64000 Pau
	Ministère des Universités, École Centrale des Arts et Manufactures, Laboratoire de Chimie Organique, Grande Voie des Vignes, 92290 Chatenay-Malabry

(*Continued on p. 78*)

TABLE 107 (*continued*)

Country	*Establishment*
	Laboratoire de Biochimie des Intéractions Cellulaires, Faculté des Sciences-Mirande, B.P. 138, 21004 Dijon Cédex
G.D.R.	Akademie der Wissenschaften der D.D.R., Zentralinstitut für Organische Chemie, Rudower Chaussee 5, 1199 Berlin-Adlershof
	Department of Chemistry, Karl-Marx Universität, Liebigstrasse 18, 7010 Leipzig
	Institut für Gerichtliche Medizin des Bereiches Medizin der Wilhelm-Pieck Universität Rostock, Runge Strasse 13, 2500 Rostock
	Karl-Marx Universität Leipzig, Sektion Chemie, Tallstrasse 35, 7010 Leipzig
Hungary	Department of Chemical Technology, L. Eötvös University, H-1088 Budapest
	Technical University, Institute of Chemical Technology, Budafoki ut 9/11, 1111 Budapest
	MÁFKI, H-8200 Veszprém
	Veszprémi Vegyipari Egyetem, Analitikai Kémiai Tanszék, Schönherz Z., u. 2, H-8200 Veszprém
	Institute for Drug Research, P.O.B. 82, H-1325 Budapest
	Budapesti Müszaki Egyetem, Biokémia és Élelmiszertechnológiai Tanszék, Müegyetem rkp 3–9, 1111 Budapest
	Research Institute for the Plastics Industry, H-1950 Budapest
	Research Laboratory for Inorganic Chemistry, Hungarian Academy of Sciences, Budaörsi ut 45, H-1112 Budapest
India	Indian Institute of Petroleum, Dehradun-248005
Iraq	Petroleum Research Centre, Council of Scientific Research, Baghdad
Israel	Department of Organic Chemistry, Weizmann Institute of Science, Rehovot
Italy	Istituto di Chimica Industriale, Università di Genova, Corso Europa 30, 16132 Genova
	Dipartimento di Chimica, Università "La Sapienza", Piazzale Aldo Moro 5, 00185 Rome
	Dipartimento di Scienca Chimiche, Università di Catania, Viale Andrea Doria 6, 95125 Catania
	Istituto di Medicina Legale e delle Assicuarizioni, Università di Catania, Via Biblioteca 4, 95124 Catania
	Dipartimento di Chimica, Università di Perugia, Via Elce di Scotto 10, 06100 Perugia
Japan	Department of Industrial Chemistry Faculty of Engineering, Kyoto University Yoshida-Honmachi, Sakyo-Ku, Kyoto-Shi, Kyoto 606
Nigeria	Chemistry Department, Bendel State University, Ekpoma, Bendel State
Poland	Department of Chemistry, Warsaw University, Pasteura 1, 02-93 Warsaw
	Technical University of Poznan, Institute of Chemical Technology and Engineering, Pl. Skłodowskiej-Curie, Poznan

TABLE 107 (*continued*)

Country	*Establishment*
	Department of Chemistry, Pedagogical University, Czestochowa
	Department of Physical Chemistry, Medical Academy, K. Marks 107, 80-416 Gdansk
	Institute of Chemistry, A. Mickiewicz University, 60-780 Poznan
Republic of South Africa	Institute for Chromatography, University of Pretoria, Pretoria 0001
Spain	Instituto Nacional del Carbon Consejo Superior de Investigaciones Cientificas, Oviedo
	Instituto de Quimica Bio-Organica (CSIC), J. Girona Salgado 18–26, 08034-Barcelona
	Centro de Investigacion Asistencia Técnica (INSHT), Dulcet s/n, 08034-Barcelona
	Instituto de Quimica Fisica "Rocasalano", Consejo Superior de Investigationes Cientificas, Serrano 119, 28006 Madrid
	Departamento de Bioquimica, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid
	Departamento de Quimica Organica, Facultad de Cienancias, Universidad de Las Islas Baleares, Palma de Malorca 07071
	Departamento de Quimica Analitica, Facultad de Ciencias, Universidad de Alcalá de Henares, Madrid
Sweden	Department of Technical Analytical Chemistry, Chemical Centre, Lund Institute of Technology, P.O. Box 740, 22007 Lund 7
	Arrhenius Laboratory, Department of Analytical Chemistry, University of Stockholm, 10691 Stockholm
Switzerland	Institut de Chimie, Université de Neuchátel, 2000 Neuchátel
	Department of Pharmacy, ETH Zürich, Zürich
	Institute of Physical Chemistry, ETH Lausanne, Lausanne
The Netherlands	Department of Toxycology, State University, Deusinglaan 2, 9713 AW Groningen
	Department of Chemistry and Chemical Engineering Organic Geochemistry Unit, Delft University of Technology, De Vries van Heystplantsoen 2, 2628 RZ Delft
	Department of Instrumental Analysis, Eindhoven University of Technology, Eindhoven
United Kingdom	Department of Chemistry and Applied Chemistry, University of Salford, Salford, Lancs M5 4WT
	DAFS, Freshwater Fisheries Laboratory, Pitlochry, Scotland
	Department of Chemistry, University of Stirling, Stirling, Scotland
	Department of Chemistry, University of Technology, Loughborough, Leics LE11 3TU
	Chemistry Department, University of Glasgow, Glasgow G12 8QQ
	Division of Chemical Sciences, Hatfield Polytechnic, Hatfield, Herts Central Research Establishment, Home Office Forensic Science Service, Aldermaston, Reading, Berks. RG7 4PN

(*Continued on p. 80*)

TABLE 107 (*continued*)

Country	*Establishment*
	Department of Chemistry, University College of Swansea, Singleton Park, Swansea, Wales SA2 8PP
U.S.A.	Sadtler Research Laboratories, Division of Bio-Rad Laboratories, 3316 Spring Garden Street, Philadelphia, PA 19104
	Department of Environmental Toxicology, University of California, Davis, CA 95616
	Department of Chemistry, Indiana University, Bloomington, IN 47405
	University of Arkansas, Fayetteville, AR 72701
	Department of Chemistry, Wayne State University, Detroit, MI 48202
	Department of Chemistry, Oregon State University, Corvallis, OR 97331
	Mesa Police Department Crime Laboratory, 130 North Robson, Mesa, AZ 85201-6697
	Perkin-Elmer Corporation, Norwalk, CT 06856
	Department of Chemistry, State University of New York at Buffalo, Buffalo, NY 14214
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20. SUMMARY

GC retention data measured in various laboratories are given in many different forms and therefore their usefulness is limited. The retention index system according to Kováts solves the problem of the uniform expression of retention data.

In this review, the main theoretical and practical results relating to retention index systems published in the past 5 years are summarized.

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REVIEW

RECENT DEVELOPMENTS IN THE GAS CHROMATOGRAPHIC RETENTION INDEX SCHEME[a]

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CONTENTS

1. INTRODUCTION

The specification of retention in gas chromatography in terms of the retention index[1], or, as it is frequently known after its founder, the Kováts retention index, has found universal application.

The index, first reported in 1958[1], found little acceptance during its first decade

[a] This work is dedicated to Professor E. sz. Kováts on the occasion of the 30th anniversary of the introduction of his retention index.

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due to its publication in German at a time when the major advances in gas chromatography were occurring in the U.K. and the U.S.A.

Despite some limitations the index has found much greater usage than all other specialised retention specification schemes, the only specialized indices of particular importance being the carbon number[2] or equivalent chain length scheme[3] developed at about the same time and used extensively with lipids and the steroid number representation used with steroidal materials[4,5].

A major impetus to the exploitation of the index scheme occurred in the U.S.A. during 1964 when at the *Second International Symposium on Advances in Gas Chromatography* at Houston both Kováts and Keulemans[6] described aspects of the retention parameter. Much of the credit for the general acceptance of the index is, however, due to the efforts of Ettre[7], who summarized in English the papers previously published in German and included details of some related schemes in a work which formed the leading article in the issue of *Analytical Chemistry* that contained the symposium proceedings.

The index has been the subject of several substantive reviews[8–10] and then of a major one by Budahegyi *et al.*[11] published in 1983 to commemorate its 25th anniversary. This exhaustive work indicated that use of the index had been reported in about 1500 publications. Many of the reports were essentially compilations of retention data whilst others related the retention index of a particular class of compounds to a wide variety of physiochemical properties. In recent years a number of minor foreign-language reviews have appeared[12–17].

The present work is much more selective than that of Budahegyi *et al.*[11] and seeks generally to include the important aspects of the index and to indicate the developments that have occurred in the last five years.

It is clear that all of the correlations of retention indices and the various physico-chemical properties are of relatively short order, or with application being restricted to a particular functional class of functional classes.

Despite much work and many reports it is obvious that no realistic scheme of wide applicability is available for the precalculation of retention indices. This problem is highlighted by several factors. Many reports employ squalane or other low-polarity stationary phases where dispersion rather than polar interactions predominate and analyte adsorption at the liquid–solid interface, a recurrent problem, occurs. With polar stationary phases many of the correlations are simply not applicable.

With the availability of computers it is frequently not too difficult to produce a mathematical relationship or equation to relate a number of experimentally determined data points. With additional data, *i.e.*, more compounds, a new relationship or one with new values for the constants is necessary. Such relationships have continued to appear and these where they contain retention data are shown in the present work in the tabulation of retention indices. Reports of an analytical nature containing retention indices of isolated compounds are generally excluded.

The effects of temperature on retention was recognized early in the history of gas chromatography and increasing the temperature during the elution sequence was soon exploited in the examination of samples with wide ranges of boiling points. With linearly increasing column temperature a nearly arithmetic rather than a logarithmic relationship, as with isothermal conditions between retention and carbon number of homologues, was evident. To facilitate retention specification with temperature-

programmed operation Van den Dool and Kratz[18] developed a programmed retention index (I_{TPGC}) using an arithmetic relationship similar to the logarithmic one of Kováts[1]. While this type of index is fundamentally different from that obtained isothermally, it has been the subject of much study due to the analytical importance of temperature programming, particularly with capillary columns. For this reason the development has continued to receive much attention and a variety of modified relationships have appeared in recent years.

As some difficulties have been experienced with the use of *n*-alkane standards, particularly with polar stationary phases, their appropriateness as standards has been questioned and a number of alternative reference series have been proposed.

Similarly, alternatives have been used with certain of the specific detectors where the alkanes do not give satisfactory responses. One situation which has received significant attention is the effect of adsorption on retention and in turn the retention index.

Other index schemes have been proposed, and both alternative standards and alternative index schemes are described in this work, neither of these areas having been described earlier by Budahegyi *et al.*[11].

In common with other areas of chromatography, it is evident that many reports, particularly from countries developing in chromatography, simply repeat results reported elsewhere years ago. Such works are generally not included in this work.

2. DEVELOPMENT OF THE RETENTION INDEX SYSTEM

The retention index (I) expresses the retention of a compound relative to homologous *n*-alkanes examined under the same isothermal experimental conditions. The retention index of a particular compound is defined as the carbon number multiplied by 100 of a hypothetical *n*-alkane having exactly the same retention characteristics, *i.e.*, adjusted or net retention time or volume or specific retention volume of the compound of interest measured under identical conditions[1].

The basic equation, first reported by Kováts and shown in eqn. 1, is based on the net retention of *n*-alkanes with an even number of carbon atoms.

$$I_S^{s.s}(T°C) = 200 \frac{\log X_S - \log X_Z}{\log X_{(Z+2)} - \log X_Z} + 100Z \tag{1}$$

where I = retention index;

S = the compound of interest;

$Z, Z+2$ = *n*-alkanes with Z and $Z+2$ carbon atoms, respectively, where Z is an even number;

X = net retention value of substance and standards used;

s.s = stationary phase used.

In fact, the values of some physical constants, *i.e.*, melting points, spectroscopic data, alternate with the odd/even series. However, soon it was realised that this is not the case for solution data, and the derivation shown in eqn. 2 was proposed[8].

$$I_s(T°C) = 100 \frac{\log X_S - \log X_Z}{\log X_{(Z+1)} - \log X_Z} + 100Z \tag{2}$$

Condition $X_Z < X_S < X_{(Z+1)}$

Subsequently, the more general expression shown in eqn. 3 was recommended by a working party of the Chromatographic Society[19].

$$I = 100N + 100n \frac{\log R_x - \log R_N}{\log R_{N+n} - \log R_N} \tag{3}$$

where R_x, R_N and R_{N+n} are the adjusted retentions of the analyte and n-alkane internal standards possessing N and $N+n$ carbon atoms, respectively. In general, eqn. 3 is a good approximation.

Both expressions are based upon the relationship between adjusted retention and carbon number for members of a homologous series of compounds shown in eqn. 4.

$$\log R = a + bN \tag{4}$$

The slope of the n-alkane log plot b, given by eqn. 5, which is determined by stationary phase polarity and mean column temperature, gives an immediate check on operating conditions[20].

$$b = \frac{\log R_{N+n} - \log R_N}{n} \tag{5}$$

For completeness the retention index should include the stationary phase used (s.s), the name of the compound of interest (S) and the temperature (°C) as shown in eqn. 1, but in practice these qualifications are rarely expressed in this way.

Kováts[1] originally suggested that three values be given: (i) the retention index at the column temperature; (ii) the retention index increment per 10°C; and (iii) the temperature range in which the index has been examined. The retention index values are directly proportional to the column temperature and with narrow temperature ranges ($\Delta T \approx 80$ K) are approximately linear although for wide temperature ranges ($\Delta T = 100$ K) the function is hyperbolic. Actually the validity of this statement depends largely on the precision of the determination.

The works of Kováts produced seven rules or relationships concerning retention index and chemical structure which also summarized regularities observed by other workers.

The first four propositions concerned the measurement of the retention index on a single phase while the remaining propositions concerned the measurement of the retention index of a particular solute on different stationary phases.

The propositions as discussed below all have been shown to be approximately true being considerable over-simplification of the actual behaviour.

(1) Within a homologous series the retention index of a higher homologue increases by 100 for each methylene unit introduced[1,21,22]. Exceptions were soon reported by Zulaica and Guiochon[23,24] who established considerably lower increments with dibasic esters. Subsequently it has been shown that few series increase by 100 units per methylene group but that most approximate to this value, *i.e.*, ±10–15%.

The particular situation with alkyl esters has been extensively studied[25] and

detailed in an earlier review[9], where it was apparent that the incremental increases varied widely. For the increments to be standardized retention plots of the homologous series must have the same slope or b value, *i.e.*, be parallel, and this does not occur.

(2) The relationship between the retention index of isomers and their boiling points was established. The differences in boiling points and retention indices of two isomers on a non-polar stationary phase such as a pure n-alkane or mixture of n-alkanes being shown by eqn. 6.

$$dI = 5dT_B \tag{6}$$

where dI and dT_B are the differences in the retention indices and the boiling points of the two isomers respectively[1,21,22]. A more complex relationship was subsequently reported between the retention index and the boiling point index by Matukuma[26] as shown in eqn. 7.

$$I_B = 10^{(0.00134052T_B + 2.558916)} - 440.5 \tag{7}$$

where I_B is the boiling point index in retention index units and T_B is the boiling point (K at 101.25 kPa).

(3) The retention index of asymmetrically substituted compounds could be calculated from the retention index values of their symmetrical counterparts[27].

(4) Similar substitution in compounds of similar structure resulted in the same retention index increase[28].

(5) The retention index of non-polar compounds (alkanes) remains almost constant for any type of stationary phase[1,21,29].

(6) The retention indices of any compound determined on various non-polar stationary phases are identical or very close to one another[1,21,22].

(7) If the retention index of a compound is determined on a polar and on a non-polar stationary phase, the difference in the retention index values, *i.e.*, ΔI, is characteristic of the structure of the compound. Furthermore, values for retention index may be calculated for a particular molecule predicted by summation of the individual increments pertaining to the various adjacent zones within that molecule[1,21,22].

The introduction of the retention index was soon followed by a number of conceptually similar representations and these, together with other indices using different calibration series for particular applications, are briefly outlined.

The theoretical nonane number or R_{x9} value was introduced by Evans and Smith[30–32] in 1960. The corrected retention of a compound was expressed relative to a computed retention for n-nonane. The linearity of the n-alkane plot was indicated together with the inter-relation of the values. The determination of retention type indices in two stages being seen at the time as something of an advantage but the scheme has found negligible acceptance.

The methylene unit scheme (MU), developed by Vandenheuvel and Horning[33] for use with biological materials, is essentially identical to the retention index scheme. A calibration line using n-docosane and n-tetracosane was used to give the methylene unit value which is merely the retention index divided by 100. The carbon number[2] and

equivalent chain length (ECL) schemes[3] employ a calibration line of the homologous methyl *n*-alkanoates. The steroid number (SN) uses a two-point calibration line of the hydrocarbons androstane and cholestane both with steroidal structures and with steroid numbers of 19 and 27, respectively[4,5], these being the number of constituent carbon atoms.

The interconversion of methods of retention presentation has long been of interest. Nine common representations of retention have been considered by Guerin and Banks[34] in 1966 and the interrelationship of equivalent chain length and retention index values has reported by Ashes *et al.*[35]. It is, of course, obvious that the universal use of minicomputers and microprocessors facilitates the interconversion of retention data.

3. ALTERNATIVES TO THE *n*-ALKANES AS CALIBRATION SERIES

Soon after the introduction of the *n*-alkanes as calibration standards alternative reference series began to be reported and this has largely continued to the present time. Certain of the alternatives have been suggested because of theoretical difficulties with the use of hydrocarbons, such as with highly polar stationary phases. Others have been necessary due to the introduction of element-specific detectors where the response of the *n*-alkanes is unsatisfactory. Sorption effects on retention indices were observed by Vandenheuvel and Horning[4] and later by Lorenz and Rogers[36], the recommendation in both cases being that the polarity of the solvent and solute should be similar. Alternative homologous series would be acceptable and be interconvertible[8], particularly if there was no adsorption at the liquid–gas interface.

An early suggestion of Dymond and Kilburn[37] was a carbon number system based on the 2-alkanones. These materials were again suggested by Ackman[38] in 1972 at a time when Grobler[39] briefly outlined the limited practicability of retention indices. Grobler highlighted the low retention values of *n*-alkanes on polar stationary phases and argued that the gas chromatography of compounds of greatly different boiling points could not be performed favourably under strictly identical conditions. *n*-Alkanols were proposed as standards but were subsequently criticized by Hawkes[40] due to the well-known anomalous effects of the hydroxyl groups. Hawkes acknowledged the limitations of simple hydrocarbons and indicated the *n*-propyl ethers as the almost ideal standards. The ether groups are in a total methylene environment uncomplicated by the effects of terminal methyl groups and display weak orientation and proton acceptor Hildebrand parameters with an absence of proton donor effects. Furthermore they are readily synthesised from the corresponding *n*-alkanols. Other available series were indicated to be unsuitable for instance the highly polar *n*-alkanols, the alkanals which have low stability and are odorous and alkan-2-ones which are not favoured due to their Hildebrand parameters.

Lower and symmetrical alkanoate esters have been evaluated as reference series[22,41] and found to be more suitable with polar solvents than hydrocarbons as suggested by Vandenheuvel and Horning[4] and Lorenz and Rogers[36]. At the time it was indicated that it was unlikely that a single reference series would ever be universally acceptable, but the difficulties were in many cases decreasing due to improvements in systems of measurement, data processing and interconversion.

Nine homologous series of compounds were examined as reference standards by

Heldt and Köser[42] using the generalised retention index developed by Novák and Růžičková[43]. These included *n*-alkanes, *n*-alkenes, *n*-alkanals, 1-chloro-*n*-alkanes, *n*-alkan-1-ols, *n*-alkan-2-ols, 1-aminoalkanes, *n*-alkylbenzenes and *n*-alkylcyclohexanes. It was found, not unexpectedly, that alkenes behaved similarly to alkanes while *n*-alkanols were unsuitable with non-polar stationary phases. The aminoalkanes were found to be unsatisfactory and the chloroalkanes of limited use with highly polar stationary phases. The *n*-alkanals were preferred as standards despite their acknowledged problems reported earlier.

Of the compounds examined[42], alcohols[43,44], ketones[44,45], esters[44] and *n*-alkylbenzenes[46,47] had been previously reported. The use of *n*-alkylbenzenes as retention standards[47] has been reported to be unsatisfactory due to poor linearity of the calibration line, which was attributed to the relative contribution of the aromatic nucleus and the methylene chain.

When stationary phases are ranked in a polarity scale, it is apparent that the particular reference standards are of major importance. With the usual situations the retention of the *n*-alkane standard largely determines the ranking. This was established 25 years ago by Littlewood[48] and was subsequently reiterated by Ashes and Haken[4] and Aue and Paramasigamani[49].

With the introduction of specialised and element-specific detectors further impetus was directed towards suitable reference standards. An early report of standards for use with electron-capture detectors was that of the *n*-alkyl iodides by Castello *et al.*[50] in 1969. With nitrogen–phosphorus specific detection a serious problem arises as the *n*-alkanes are virtually non-detectable. Attempts at overcoming the problem have included (a) the use of excessive injection of *n*-alkanes to achieve a slightly greater response[56], (b) the use of a mixture of nitrogen-containing compounds (frequently drugs) as secondary standards with published retention index values instead of the *n*-alkanes[51] or (c) use of relative retention times based on nitrogen-containing drugs as internal standards instead of the Kováts procedure using *n*-alkanes[52,53].

The use of excessive amounts of *n*-alkanes with nitrogen–phosphorus detection can lead to variation of the retention time, peak broadening, peak tailing and peak splitting[54].

A method developed by Asselin[55] allows the detection of compounds such as the *n*-alkanes which do not contain phosphorus or nitrogen using a thermionic phosphorus–nitrogen specific detector. The method requires the temporary conversion of the nitrogen–phosphorus detector into a detector with properties approximating those of a flame ionization detector. The hydrogen flow-rate was deliberately increased from its normal flow-rate (2.0–4.0 ml/min) to 8 ml/min where the *n*-alkanes are readily detected. After this calibration has been achieved the gas flow-rate is returned to its normal level to allow nitrogen–phosphorus detection. A reconditioning period of about 45 min is necessary to ensure that the ceramic bead of the detector is sufficiently stable for quantitative measurements.

During the 1980s there has been a very large number of alternative reference series proposed for use with the electron-capture and specific-element detectors particularly for nitrogen, phosphorus- and halogen-containing materials, drugs, polychlorinated biphenyls and other aromatic compounds, which have become of considerable importance.

The symmetrical *n*-dialkyl (C_{4-12}) sulphides were examined by Zotov *et al.*[56] for use with detectors insensitive to *n*-alkanes. Retention indices (I_S) were obtained isothermally and also by temperature programming using the equation of Van den Dool and Kratz[18]. It was reported that the dialkyl sulphides follow the same laws as the *n*-alkanes and are satisfactory as standards.

A multidetection retention index scheme has been developed by Enqvist *et al.*[57] involving the use of *n*-alkyl bis(trifluoromethyl)-thiophosphinates as internal standards in connection with element-specific assays. The multifunctional standards enable the measurement of a form of retention index with single or dual-channel instruments equipped with flame ionization, electron-capture, nitrogen–phosphorus and flame photometric detectors. With the latter of single-column instruments equipped with an appropriate effluent stream splitter, the combination of element-specific and flame ionization detection can yield conventional retention indices by the conversion of relative retention ratios by means of the expression[58] shown in eqn. 8.

$$I = 100 \frac{\log R_{xS}}{b} + I_S \tag{8}$$

where R_{xS} is the retention of the unknown relative to the multifunctional standard, obtained by element-specific detection, and I_S is the retention index of the *n*-alkyl bis(trifluoromethyl)thiophosphinate standard obtained by flame ionization detection.

The *n*-alkyl trichloroacetates were described as retention index standards by Neu *et al.*[59] in 1978 who also reported their use in several works concerning the analysis of polychlorobenzenes and polychlorinated biphenyls[60,61].

Analysis of polychlorinated biphenyls was also carried out using *n*-alkyl trichloroacetates as standards by Schwartz *et al.*[62]. Three esters were used, namely the *n*-decyl, *n*-dodecyl and *n*-pentadecyl. A computer-assisted technique for the quantitative determination of many of the 209 theoretically possible polychlorinated biphenyl isomers was subsequently described by the same workers[63]. The *n*-C_{8-20} alkyl trichloroacetates were used as external retention standards by Wegman and Hofstee[64] for analysis of polychlorinated biphenyls in aquatic sediments using temperature programming.

A method for the calculation of retention indices using a power series expansion of the logarithmic relationship between retention and carbon number was reported by Heeg and Zinburg[65]. The trichloroacetates were indicated to be a suitable reference series for compounds sensitive to electron-capture detection.

The monobromoalkanes have continued to be recommended as retention calibration markers[44,50,66] for use in both the isothermal and temperature-programmed mode.

The retention index shown as I' of 221 halogenated aliphatic and alicyclic compounds have been reported on the 1-bromoalkane scale by temperature-programmed capillary gas chromatography by Yasukara *et al.*[66] while elemental iodine was determined with electron-capture detection using retention indices based on *n*-alkanes and alkyl iodides. The iodine passed through the column without conversion to an organic halide with concentrations of 118 mg/ml being determined[67].

Pacholec and Poole[68–70] used a homologous series of *n*-bromoalkanes to produce a retention index scale and a novel calibration method for use with electron-capture detection. The response per mole of the detector is identical for all the

C_{5-18} *n*-bromoalkanes. An internal calibration curve was obtained for each sample injected by adding several *n*-bromoalkanes the molar concentrations of which are related in a simple step wise fashion to one another. This calibration method is capable of a relative average percentage error of 4–10% compared with an error of 12–20% with the standard calibration method for concentrations above the detection limit. The method with *n*-alkyl iodides[70] was applied to temperature-programmed capillary gas chromatography. Retention indices were reported to be reproduced with an average standard deviation of approximately 0.35 index units with the relative error for the method being similar to that achieved by daily solute calibration.

A chromatographic retention index (I_N) based on homologous tri-*n*-alkylamines has been devised for use in the detection of pesticides and related compounds in body fluids of agricultural workers[63]. The amines tri-*n*-propylamine and tri-*n*-decylamine were assigned index values of 300 and 1000 according to the number of carbon atoms in a single alkyl chain. Capillary column studies using dimethyl polysiloxane produced index values for 106 pesticides and related compounds. The relationship between these indices and those of Kováts was examined[71]. A subsequent capillary column study[72] of 152 drugs and metabolites was carried out using 5 and 50% phenyl-substituted polysiloxane as stationary phases. The retention indices were obtained by increasing the hydrogen and air flow-rates essentially as indicated by Asselin[55].

An index system suitable for electron-capture detection in the examination of pesticides and environmental compounds by temperature-programmed capillary gas chromatography has been developed using chlorinated compounds such as chlorobenzene isomers and decachlorobiphenyl. The scheme described as the chlorophenyl (CP) index used 1,3,5-trichlorobenzene, 1,2,4,5-tetrachlorobenzene, hexachlorobenzene and decachlorobiphenyl as standards with assigned values of 300, 400, 600 and 1000, respectively[73].

Nakamura[74] has examined, with environmental samples, sulphur-containing heterocyclic compounds and compared the polyaromatic hydrocarbon (PAH) index, the retention index and the CP index.

Polychlorinated biphenyls have been obtained for use as retention index standards by the partial catalytic dechlorination of polychlorinated biphenyls having up to six chlorine atoms. The dechlorination employed $NaBH_4$ and $NiCl_2$ catalysts generated *in situ*, and all the expected polychlorinated biphenyl congeners were observed[75].

A study of the retention indices of PAHs on a low-polarity capillary column (SE-52) showed poor statistical reliability. A new retention index described variously as the PAC index, the PAH index or the Lee index was developed based on a series of PAHs[76].

The standards naphthalene, phenanthene, chrysene and picene were assigned values of 200, 300, 400 and 500, respectively, with the system having no foundation in chromatographic thermodynamics. It does not assume a linear relationship between retention and ring number or other structural parameters and uses the linear equation of Van den Dool and Kratz[18].

The retention indices of more than 200 PAHs were determined with the average 95% confidence limits for four determinations on each PAH being ± 0.25 index units[76]. A subsequent work[77] evaluated the results of 29 routine capillary gas chromatographic analyses of complex mixtures of polyaromatic compounds. The

temperature-programming rates and the effect of initial isothermal periods were examined and tabulations of additional polyaromatic compounds, including 78 hydrocarbons, 115 sulphur and 117 nitrogen heterocyclic compounds included.

The PAH index has been applied to a considerable number of related studies of: diesel particulate matter[77,78], coal extracts[79], synthetic fuels[80,81], tissues and sediments[82–85], air particulate matter[86] and combustion effluents[87].

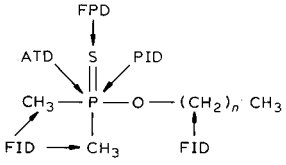
Rostad and Pereira[88], using gas chromatography–mass spectrometry of environmental samples, determined PAH indices and modified Kováts indices (I_{TPGC}) for a wide range of aromatic and aliphatic compounds.

The PAH index system has been applied to an automated system for the analysis of products generated by coal gasification in a two-stage fixed-bed gasifier system[89]. A similar system using both gas chromatography and gas chromatography–mass spectrometry for the same application has been reported by Stamouis and Demirgian[90].

Several specialised series of compounds have been synthesized for application with the newer specific detectors.

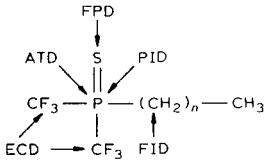
A homologous series of 2,4-dichlorobenzyl alkyl ethers (C_{2-16}) was synthesized and purified for use as retention index standards. The compounds are stable, sensitive to electron-capture and flame ionization detection and suitable for gas chromatography–mass spectrometry with base peaks of m/z 159/161. On fragmentation good total ion current detection occurs with an intense ion at an $m/z > 100$ above the low mass background with suitability for single or multiple ion monitoring[91,92].

Of two related series the first described as the P series by Manninen *et al.*[93] are alkyl dimethylphosphinothioates, which possess functional groups suitable for use with flame ionization, nitrogen–phosphorus and flame photometric detection, but not electron-capture detection.



ATD = alkaline thermoionization detection
FPD = flame photometric detection
FID = flame ionization detection
PID = photoionization detector

Subsequently, a series of alkyl bis(trifluoromethyl)phosphinesulphides were introduced (designated the M series) that are suitable also for use with electron-capture detection[94].



ECD = electron-capture detection

The components of the M series were detected at the level of 0.4–0.6 pmol with flame ionization detection, 2–7 fmol with nitrogen–phosphorus detection and 305 fmol with electron-capture detection. The sensitivities of nitrogen–phosphorus and elec-

tron-capture detection are so much greater than flame ionization detection that their parallel use with the two-channel chromatograph was not possible.

The components of the M series are comparatively non-polar on the non-polar (SE-54) stationary phase and differ little from those of the *n*-alkanes. A linear correlation was shown between the retention indices of nine different chemical agents based on the *n*-alkanes and the M series. The M series standards also have been used in a multidetector system[57].

It is clear that while a considerable number of homologous series have been reported as retention standards there is little likelihood of a universally acceptable series ever being established. With many of the specialised series higher homologues are involved than with the *n*-alkanes. In these cases, *i.e.*, fatty acids, the difficulties of deviation from linearity of the lower members in the semi-logarithmic plot of retention and carbon number are not experienced.

4. LOGARITHMIC INDEX SCHEMES

A number of index schemes very similar to the retention index have been reported and are described below.

Considering the thermodynamic derivation of the retention index Heeg and Zinburg[65] have shown, as have many other workers, that the linear relationship between the logarithm of the net retention time and the carbon number of homologous series is valid only to the first approximation. The authors reported that with accurately measured solute values a more exact description of their function is achieved by the use of a power series of higher order as shown in eqn. 9.

$$\log t'_R = \alpha + \beta c + \gamma c^2 + \delta c^3 + \ldots \tag{9}$$

where t'_R is the adjusted retention time, c is the carbon number and α, β, γ, δ, etc. are constants. These considerations led to a method for the determination of retention indices which by virtue of the more vigourous treatment are reported to make possible extrapolation over a wider range and similarly wider interpolation.

4.1. Generalised retention index

The generalised retention index $I^x_y(i)$ was developed by Novák and Růžičková[43] in 1974 and is a simple extension of the Kováts index which allowed the retention index to be applied to other homologous series of standards. The index is shown in eqn. 10.

$$I^x_y(i) = \frac{100(\log V^x_{N(i)} - \log V^x_{N(yn)})}{\log V^x_{N(yn+a)} - \log V^x_{N(yn)}} + 100_n + Ky \tag{10}$$

where $V^x_{N(i)}$, $V^x_{N(yn)}$ and $V^x_{N(yn+a)}$ are the net retention values on a stationary phase x of the substance examined, and of the reference compound with N, and $(N+a)$ methylene units, respectively. Ky is a constant selected to avoid negative indices where with *n*-alkanes $Ky = 0$.

The scheme was subsequently evaluated by Heldt and Köser[42] using nine homologous series of varying functional class, most of which had been considered previously by other workers.

The same name, *i.e.*, generalized retention index, has been given to a scheme by Zenkevich[95]. A new algorithm for calculating retention indices is based on a linear relationship between I and $(t'_R + q \log t'_R)$ where t'_R is the adjusted retention time and q is a coefficient related to the analytical conditions. The generalized retention index values combine the system of logarithmic Kováts indices and linear indices. The generalized retention index under any conditions of temperature programming can be compared with the normally isothermally determined Kováts index.

The Kováts equation as shown in eqn. 11 is a logarithmic relationship of retention and carbon number.

$$\log R = a + bN \tag{11}$$

The linear equation of Van den Dool and Kratz[18] may be shown as

$$R = a' + b'N \tag{12}$$

where N is carbon number and a, a', b and b' are constants.

4.2. Homologous index

The same idea as the generalised retention index termed the homologous index (I_h) has recently been described by Luo *et al.*[14]. This is defined as a retention index obtained by using the homologous series of the substance to be characterized by gas chromatography as the reference series. Based on the analysis of hierarchial data with five sources of variation, the relative standard deviations of the proposed homologous retention index caused by the concentration of stationary phase and column temperature are much smaller than those of relative retention and Kováts retention indices. The homologous retention index is claimed to be more precise and reliable for characterization. The retention index is considered to be a special case of the homologous retention index when the *n*-alkanes are used as standards.

The conclusions of the authors that it is preferable to use standard similar to the compounds examined are as previously reported by other workers, both with regard to the characterization of solutes and solvents.

4.3. Unified retention index

The unified retention index developed by Dimov[96] has been used to explain the variations in the retention index of simple hydrocarbons on squalane which are largely attributed to random errors. The temperature dependence of retention index is well known, the function $\mathrm{d}I/\mathrm{d}T$ being hyperbolic. A statistical treatment using simple regression analysis of the data allows computation of a unified retention index (UI_T) as shown in eqn. 13.

$$UI_T = UI_0 + (\mathrm{d}UI/\mathrm{d}T)T \tag{13}$$

where UI_0 is the value of UI_T at 0°C and $\mathrm{d}UI/\mathrm{d}T$ the temperature dependence where $-\mathrm{d}UI/\mathrm{d}T$ is the slope of the plotted data.

Tabulated data were presented for many hydrocarbons on a squalane column[96] and subsequently on dimethylpolysiloxane (OV-101) between 40° and 70°C[97] and later

for aromatic hydrocarbons on squalane[98]. An algorithm has been reported for the precalculation of the optimum separation for the gas chromatography of a petroleum fraction. The algorithm is based on two concepts, the unified retention index and a mathematical description of the dependence of the peak width and retention indices and on the column temperature[99].

The retention of the hydrocarbons present in the C_5 pyrolysis fraction of gasolines on squalane and dimethylpolysiloxane oil (J × R) capillary columns was investigated. The unified retention indices of the hydrocarbons were determined on squalane. The retention indices obtained on the two phases were interrelated. Equations based on the unified retention indices on squalane allowed calculation of the values on dimethylpolysiloxane with reasonable accuracy[100].

4.4. Standard retention index

Robinson and Odell[101] in 1971 proposed the standard retention index scheme I_{STD} using the equation developed by Kováts[1] but considering the boiling points of the substances as shown in eqn. 14.

$$I_{STD} = 100n + 100 \frac{\log BP_x - \log BP_n}{\log BP_{n+1} - \log BP_n} \tag{14}$$

where BP_x, BP_n and BP_{n+1} are the boiling points of the compound of interest and of the *n*-alkanes with n and $n+1$ carbon atoms respectively. A standard retention index difference (ΔI^*) was obtained by comparison of the standard and experimental retention indices.

$$\Delta I^* = I_{STD} - I_x^T$$

where I_x^T is the retention index of the compound at temperature T on the stationary phase x. The method has found no acceptance and Dimov[102] in an evaluation found the accuracy to be low.

4.5. Invariant retention index

The effect of the solid support on the chromatographic separation has long been realised especially by Berezkin[103,104], who developed the relationships shown in eqns. 15–17.

$$I = I_0 + a/P_L \tag{15}$$

$$I = I_0 + b/k_s \tag{16}$$

$$I = I_0 + c/V_L \tag{17}$$

where I_0 is the invariant retention index;

a, b and c are constants;

P_L is the percentage stationary phase on the solid support;

V_L is stationary phase volume in column;

k_s is the capacity factor for the reference compound, the retention of which is mainly determined by its solubility in the stationary phase.

It was found preferable[105] to use the equation containing the capacity factor, as the equation with the stationary phase loading is of low precision due to its measurement with losses that are experienced during coating. Similarly, the relationship with the capacity factor allows calculation of the invariant retention indices on capillary columns with small errors[106,107].

The invariant indices based on the *n*-alkanes, *n*-alkan-1-ols and *n*-alkan-1-ol acetates were reported for insect sex pheromones and their structural analogues belonging to a series of saturated and unsaturated alcohols and esters. The homologous series of alkanols and esters were found to be the most suitable standards for determinations with the minimum errors.

The interlaboratory reproducibility of gas chromatographic data was reported to be improved by determination of invariant indices. Studies were carried out using squalane for the separation of hydrocarbon mixtures to demonstrate the difference between conventional and invariant retention indices[108].

A linear relationship between retention indices and the film thickness of the stationary phase (PEG 20M) in capillary columns was reported[109].

The use of the invariant indices in the calculations increased the reproducibility in the determination of the retention indices when different operators and different laboratories were involved. The invariant index is reported to be independent of the column packing material.

4.6. Universal retention index

It has been reported by Belyaev and Vigdergauz[110] that the reference solutes should not be identical in chemical nature, for instance as with the *n*-alkanes, but rather identical with respect to the degree of sorption on different stationary phases, *i.e.*, hypothetical compounds with fixed values of the distribution coefficient (or retention volume) which do not alter when going to different phases.

Values of selectivity coefficients equal to the ratio of the reduced retention times of neighbouring homologues on non-polar stationary phases near 100°C where the polarity factor N_u is approximately 2. It is proposed that the universal series be a hypothetical reference series where retention is determined from the relationship shown in eqn. 18.

$$V_{g(N)} = 2^{N-1} \tag{18}$$

Where $V_{g(N)}$ is the absolute specific retention volume of N, the hypothetical compound, the first member of the series being assigned $V_g = 1$ cm^3/g (close to V_g of methane on non-polar phases at 100°C).

The retention of each subsequent member will be twice as great as the preceeding one hence the reduced retention time (t'_{RN}) of the hypothetical reference compound, NH, is given by the expression shown in eqn. 19.

$$t'_{RN} = \frac{2^{N-1} gT}{FJ \cdot 273.15} \tag{19}$$

where g is mass of stationary phase (g);
T is column temperature (°C);
F is flow-rate (ml/min);
J is pressure gradient factor.

A comparison of nine methods for determining the universal retention index has recently been reported by Belyaev and Vigdergauz[111] using both experimentally determined and published retention data. The valuations were carried out using alkanes, alcohols and aromatic hydrocarbons. Chromatography was performed at 120°C using a liquid crystal stationary phase (*p,p'*-ethoxypropoxyazobenzene).

4.7. Molecular retention index

The molecular retention index (ΔM_e) was developed by Evans and Smith[112,113] in 1961 as an alternative to the retention index. The index is similar to the Kováts scheme[1] and is defined as the molecular weight of a hypothetical *n*-alkane having the same retention as the compound under study. The formula of the hypothetical *n*-alkane equivalent to the compound is $C_{I'}H_{2I'+2}$ and will have an effective molecular weight given by the relationships shown in eqns. 20 and 21.

$$M_e = 14.026\ I' + 2.016 \tag{20}$$

$$= 0.1406\ I + 2.016 \tag{21}$$

where $I = 100\ I'$;

M_e = effective molecular weight of the solute or the molecular weight of a hypothetical *n*-alkane with I' carbon atoms;

I' = number of carbon atoms in the hypothetical alkane;

$\Delta M_e = M_e - M$;

M = the actual molecular weight.

The difference between the effective and true molecular weights of the compound were suggested to be a useful parameter for the correlation of chromatographic retention and chemical structure.

The scheme was subsequently developed by Evans[114] and has been suggested for stationary phase characterization without the necessity for using the unstable squalane base stationary phase[115,116].

The scheme has found negligible acceptance, nevertheless such relationships have subsequently been reported by several workers. Piringer *et al.*[117] in 1976 described a method in which combinations of retention increments, compatible with the empirical formula and the retention of an unknown compound are used for its identification.

A parameter W was determined as shown in eqn. 22.

$$W = M_e - M \tag{22}$$

$$= 0.14I - M + 2$$

where M = molecular weight;

M_e = molecular retention index;

I = retention index.

More recently a similar scheme has been detailed by Zenkevich and Malamakhov[118] in 1987; however, in common with the previous molecular retention index systems it has found little application.

4.8. Dispersion and selectivity indices

While it has been considered that the molecular retention index (ΔM_e)[112,113] has some advantages over the retention index for the characterization of both solvents and solutes, it has not found acceptance. Accordingly a scheme was developed[119] whereby the retention index and molecular retention relationships were combined hopefully to extend the utility of the almost universally used retention indices.

For a particular solute the partition coefficient is determined by the magnitude of the intermolecular forces involved in its interaction with the stationary phase. With *n*-alkanes these forces are almost exclusively London dispersion forces, which are additive and increase with molecular weight. As heteroatoms and multiple bonds are introduced into a molecule, polar forces contribute increasingly to the overall retention. It is these forces, moderated by the influence of molecular shape or steric effects, that account for selectivity.

On the assumption that the non-polar forces involved in retention are directly proportional to the molecular weight, the retention index may be shown by eqn. 23.

$$I = I_M + I^* \tag{23}$$

where the dispersion index I_M is defined as the retention index of a hypothetical *n*-alkane having the same molecular weight as the solute; I^* = selectivity index; and I = retention index.

When I_M is defined as in eqn. 23 its value may be determined according to the relationship shown in eqn. 24.

$$I_M = \frac{M - 2.016}{0.14026} \tag{24}$$

where M = molecular weight. The retention index $I = 100i$ where i is the carbon number of hypothetical *n*-alkane C_iH_{2i+2} or $(CH_2)_i + 2H$, with a molecular weight of $14.026i + 2.016$. By definition $I_M = I$ for *n*-alkanes, the difference I^* of the solute reflecting the combined polar interaction.

The selectivity index I^* is the carbon number equivalent of ΔM_e given in eqn. 25.

$$\Delta M_e = M_e - M \tag{25}$$

where M_e = molecular retention index arising from the logarithmic relationship between the retention of the *n*-alkanes and molecular weight.

M = molecular weight of the particular solute.

It has been shown that solutes possessing polar functional groups tend towards positive I^* values whereas chain branching and substituents with screened electrons yield negative values.

The scheme has recently been demonstrated using a large number of homologous series of halogenated cyclic compounds[120], alkyl- and alkenylbenzenes[121], aliphatic[122] and aromatic[123] esters and halogenated aromatic compounds[124] and also for stationary phase characterisation[125].

A similar concept recently has been applied to liquid chromatography by Yamaguchi and Hanai[126]. The retention of a molecule in high-performance liquid chromatography was considered to be determined by various intermolecular interactions, dispersion, inductive, orientative and charge-transfer including hydrogen bonding. Dispersive, inductive and orientative interactions additively can be related to molecular size (Van der Waals volume). On the other hand, the selectivity of retention difference may be related to charge-transfer interactions, including hydrogen bonding. The equation proposed by Evans *et al.*[119] was modified to give eqn. 26.

$$R = R_v + R^* \tag{26}$$

where R_v, the molecular size index, is defined as the retention index of a hypothetical *n*-alkane having the same Van der Waals volume as the analyte and R^* is a selectivity index similar to the energy effect in reversed-phase liquid chromatography[127,128].

5. LINEAR INDICES

A simple linear index scheme (J) was reported by Vigdergauz and Martynov[129,130] using the uncorrected retention of the *n*-alkanes as a basis for interpolation according to eqn. 27.

$$J = \frac{d_{\text{substance}} - d_{n-c_Z}}{d_{n-c_{Z+1}} - d_{n-c_Z}} \tag{27}$$

Where $d_{\text{substance}}$, d_{n-c_Z} and $d_{n-c_{Z+1}}$ are the gross retention values of the substance and *n*-alkanes with Z and $Z+1$ carbon numbers, respectively. An identical relationship was introduced about the same time by Harbourn[131] who stressed the simplicity of its use, nevertheless the scheme has found little usage in isothermal gas chromatography[132,133]. It is evident that such uncorrected indices are of negligible interlaboratory use and have little theoretical basis in chromatographic thermodynamics. Such linear indices have found widespread use in temperature programmed gas chromatography. It was early recognised that with a linear temperature increase a near linear relationship existed between the retention of homologues and their carbon number. The equation of Van den Dool and Kratz[18] providing a temperature-programmed version of Kováts indices shown as I_{TPGC} was developed in 1963. And since this time with the extensive acceptance of temperature-programmed operation such linear indices have received much attention. Such indices, however, have little basis in theory and are found to be much more variable than isothermal results, particularly on an interlaboratory scale. The very many conflicting works that have appeared on the topic are not include here.

With programming of the carrier gas, *i.e.*, flow programming, very minor variations of the retention indices only are experienced; however, the technique while developed decades ago has found negligible acceptance.

6. DEAD TIME CONSIDERATIONS

The estimation of the dead time or gas hold-up time corresponding to the instrumental contribution to the gross retention is of considerable importance in the accurate determination of the retention of a compound, and similarly to any subsequently calculated retention index.

The major interest in dead time determination during the last decade has been its measurement mathematically. This is conveniently carried out by linearisation of the alkane calibration plot where a single injection of the homologous *n*-alkanes provides the dead time and the adjusted retention times of the *n*-alkanes for use in the determination of the retention index.

Because of the widespread use of on-line data processing the topic has been extensively studied and has been the subject of several reviews[134–136] and reports comparing the effectiveness of some of the principal procedures. These works have essentially included all the published work in the area and the details are not repeated here.

The use of linearisation of the *n*-alkane line depends on a linear relationship existing between carbon number and retention. This has been a contentious issue for years and it is now evident in most if not all cases that at least some minor deviations exist with the lower members of a homologous series[137]. A widely used procedure being to avoid the use of the lower homologues with the values being readily calculated mathematically. The area has also been examined by Riedo *et al.*[138] who showed that the relationship between the carbon number and logarithm of the net retention was not strictly linear. As an alternative they presented simple methods for the determination of the dead volume using flame ionisation and thermal conductivity detection.

While methane was unsatisfactory it does not give a value near the true zero. The true zero was determined from the relative retentions of a pair of substances determined with the necessary precision. Methane was one substance selected and was injected together with a second compound A, which is sufficiently separated from methane. The relative retention of A with respect to methane is then given by eqn. 28:

$$r_{A/CH_4} = \frac{X'_R(A)}{X'_R(CH_4)} = \frac{X_R(A) - X_m}{X_R(CH_4) - X_m} \tag{28}$$

where X is the retention property of the solute (distance, d; time, t; volume, V) and r_A, X_R and X_m represent the gross, corrected (net) and dead retention properties, respectively. Eqn. 28 gives, after the necessary transformations:

$$X_m = X_R(CH_4) - \frac{X_R(A) - X_R(CH_4)}{r_{A/CH_4} - 1} = X_R(CH_4) - \delta \tag{29}$$

For calculation of the value of δ, a precise knowledge of the relative retention r_{A/CH_4} is necessary.

A chromatograph fitted with a thermal conductivity detector and a hydrocarbon ($C_{87}H_{176}$) column was used for determination of the gross retention volumes of a series of permanent gases and light hydrocarbons. The results showed neon to have

the smallest gross retention time and thus accepted as the dead volume. With a thermal conductivity detector, nitrogen was accepted as a non-retained substance.

Various alternative homologous series have been used for the determination of the dead time. An early work considered C_{11-15} methyl *n*-ketones, C_{10-20} methyl *n*-alkanoates, C_{10-20} *n*-alkanols, and C_{12-24} *n*-alkanes where it was concluded that little difference was experienced with the various systems[139]. A more recent work[47], however, showed that the *n*-alkylbenzenes were unsuitable for the purpose of dead time calculation in gas chromatography.

The use of methane as void volume marker, first proposed by Feinland *et al.*[140], is frequently used in retention index measurements. However, although convenient the method is suspect because methane is known to be retained significantly on apolar columns at moderately high temperatures[141]. An observation which is at variance with Kováts' proposal that assumes a value of 100 for methane whilst as a valid dead time calibrant a value of zero is necessary.

Parcher and Johnson[142] also used inert gases to calculate the dead time and noted that methane did not fit on the linear semilogarithmic plot of retention and carbon number. They reported an effective carbon number of 0.5 for methane. A wider range of stationary phases was subsequently used[143] to investigate the effective carbon number as mathematically the deviations from linearity on a plot appeared small. Wide variations in the effective carbon number of methane were observed on the various phases but in all instances the values were substantially closer to unity than previously reported.

It is of course apparent that the effect of variations in the determination of dead line are more serious with compounds of lower retention however with the very widespread use of computerised systems the procedure adopted should be as rigid as possible. While the equations of Kováts were reported long before the use of computerised systems two problems have been observed in the mathematics involved. Alternative mathematical procedures have been discussed to remove these difficulties[144].

7. REPRODUCIBILITY OF RETENTION INDICES

Retention indices may be reproduced within a laboratory using modern instrumentation with considerable precision over finite time periods, *i.e.*, often short, depending on the stability of the stationary phase. Reproducibility of 0.05–0.1 units was reported by Schomberg and Dielmann[145] in 1973 and such results were routinely obtained in studies of McReynolds constants at about the same time. However, columns with sensitive packings such as the squalane standard produced reproducible results for only a few hours and therefore required to be continually replaced. For routine operation a reproducibility of about one index unit might be expected with a non-polar phase and 2–3, possibly 5, units with a highly polar phase.

Errors that occur in retention index determination may be random or systematic[145–148]. Random errors fall within two areas, namely errors in measurement of the elution time and variations in elution time that occur due to small alterations in the instrumental parameters, *i.e.*, oven temperature, gas flow. While elution time is now accurately measured and operating conditions are more closely controlled it is difficult to ensure that such errors are eliminated. The chromatographer has little

control over such errors which are largely characteristic of the instrumentation used. Systematic errors are caused by many factors although the stability of the stationary phase is probably the most serious cause followed by solute adsorption at the solid–liquid interface. While some improvements in these areas are possibly by the chromatographer it has been suggested that it is not possible to ensure their complete elimination as no acceptable monitoring procedure is available[147].

The performance of a material as a stationary phase apart from a suitable physical form depends on its chemical nature, *i.e.*, chemical composition, and obviously to obtain reproducible results this must remain unchanged. Large numbers of reports over decades have appeared concerning the thermal stability and variability of composition of stationary phases and while improved products have been available in recent years, considerable further improvement is possible.

While attention has been directed to the stationary phase the packing of a conventional column or coating of a capillary column is equally of importance in influencing operation and achieving reproducible results. Many phases are susceptible to various solvents and care must be exercised from the commencement of preparation. With packed columns the inertness of the support is significant as is deactivation of the inner surface of capillary columns.

Many phases contain low-molecular-weight compounds which tend to bleed away in use and alter the chemical composition of the phase and accordingly its column characteristics. The majority of phases are polysiloxanes which usually contain small amounts of cyclic and linear oligomers or reactive intermediates, depending on the method of synthesis, as well as catalyst residues from the syntheses. With immobilised phases cross-linking catalyst residues frequently remain while with many early laboratory prepared columns the degree of cross-linking varied slightly with each column as indicated by the varying amounts of materials leached away by solvents. All of these compounds present in a phase are of slightly different polar character and effect the overall retention characteristics of the material. As all of these components are lost or vary in composition with column use the retention characteristics as a consequence continually drift. With the increasing use and availability of commercially prepared columns some of these difficulties are reduced.

Peak tailing is a fairly common phenomenon and is frequently due to adsorption of the solute on the support. With significant tailing the elution pattern is altered. In gas chromatography, separation and elution are due to a partition mechanism while adsorption introduces a second mechanism and accordingly a composite mechanism is in operation. Supports have been even more variable than stationary phases and the adsorption contribution to elution should be minimal to ensure reliable peak identifications.

Adsorption has been suggested as the principal cause of poor inter-laboratory reproducibility of retention data[149] the effect is most apparent with non-polar phases where the polar contribution of the phase is low and that of the support relatively high. The increased effect of adsorption with decreasing stationary phase film thickness having been demonstrated by Krupčik and co-workers[150–151].

8. GAS–LIQUID INTERFACIAL ADSORPTION AND RETENTION INDICES

The effect of this adsorption has long been recognised with an equation relating gas chromatographic retention and various partition coefficients being reported in 1961 by Martin[152]. The equation was subsequently extended by Berezkin[149] and Martire[153] and gives the net retention volume as the sum of four terms concerning (a) dissolution of the solute in the bulk of the liquid film (b) adsorption at the surface of the liquid (c) adsorption at the liquid–solid interface (d) adsorption of the solute at the non-wetted surface of the support. Adsorption has been described in appropriate texts[154] and reviews[153], and the effects only are included in this work.

Non-polar solutes and strongly polar solvents exhibit strong adsorption as they are of limited compatibility. An excess surface concentration of solute occurs as it is largely excluded from the bulk of the stationary phase. The effect also may occur with the opposite situation as observed by Martin and Gump[155] as demonstrated by squalane and polar solutes.

The Gibbs surface adsorption is a complication in retention measurement and while it may not be eliminated it may be minimised by the use of small solute samples as it is concentration dependent[154].

9. RETENTION INDEX DETERMINATION WITH COMPUTERS AND COUPLED INSTRUMENTS

The universal availability of mini-computers and microprocessors and of coupled and multi-detector instruments have continued to be reported as means of on-line and off-line determination of retention indices and or compound identification. Such methods as have been reported in recent years are usually not novel and accordingly are only briefly indicated in this work.

The automatic calculation of retention indices using both on-line and off-line operation with computing devices from simple calculators have been described by various workers[156–160], but are readily available as turn-key systems from the major instrument suppliers. Several systems of a process or pilot plant nature for the measurement and estimation of the hydrocarbons from the gasification of coal have been described[89,90]. The automatic gas chromatographic evaluation of the performance of membranes has been reported[161].

Multi-channel and multi-detector systems have also been reported[162–165] although such systems have been available for decades. Computerized gas chromatography[166] and gas chromatography–mass spectrometry[167,168] instruments have been described as have searching techniques of retention indices and mass spectra[169–171]. A retention index library of capillary chromatographic data has been developed by Sadtler Laboratories[172] and its use reported in micropollution analysis[173,174].

10. RELATIONSHIP BETWEEN RETENTION INDEX AND PHYSICO-CHEMICAL PARAMETERS

Throughout the history of chromatography there has been continued interest in relating retention parameters, including retention index, with physico-chemical quantities. The difficulty with all of these relationships is that they are restricted to

TABLE 1

RECENT STUDIES ON RETENTION INDICES AND PHYSICO-CHEMICAL QUANTITIES

Property	*Ref.*
Boiling point	179–183
Density	184
Molar mass	185
Vapour pressure	185
Molar volume	186–188
Refractive index	182, 185, 187, 189
Molar refraction	181, 186–191
Thermodynamic parameters	192–195
Van der Waals volumes	182, 187, 196, 197
Dipole moment	180, 188
Capacity factors	198
Cohesion parameters	199
Electric interactions	200–202
Molecular orbitals	203
Taft and Palm constants	204
Molecular polarizability	205
Solubility factors	206
Connectivity index	182, 191, 196, 204, 207–211

a single or similar functional class with the data generally being obtained on non-polar stationary phases where the operative interactions forces are large dispersion, On polar stationary phases many of the simpler relationships are found to be non-linear.

Many workers have studied generally the various physico-chemical quantities on small groups of compounds[175–178]. Many of the widely discussed parameters —boiling point, density, refractive index, molar refraction, dipole moment, vapour pressure— have continued to attract attention while the area of most development concerns the relationship of retention indices and connectivity indices. Representatives of the reports that have appeared are shown in Table 1 with the parameters of interest being indicated.

An extremely important and simple relationship was developed by Kováts and Weisz[212], but has found little use. The retention index was shown to be related to the standard chemical potential of the solute between the ideal gas state and the ideal dilute solution if there is no adsorption at the gas–liquid interface. The relationship is shown below as eqn. 30.

$$I = 100 \frac{\Delta\mu_s - \Delta\mu_z}{\Delta\mu_{Z+1} - \Delta\mu_Z} + 100Z \tag{30}$$

where $\Delta\mu$ is the standard chemical potential difference between the gas phase and the solution.

An extension of the method of Laffort and Patte[213] for the calculation of solubility factors in gas chromatography has recently been refined[206] and applied to 240 compounds covering a wide range of functional classes and structures. The retention index is related by the expression shown in eqn. 31.

$$I = \alpha A + \omega O + \varepsilon E + \pi P + \beta B + 100 \tag{31}$$

where the solubility factors are shown by the greek letters and the stationary phases by the arabic letters. The determination of liquid–liquid partition data has been reported using the 45 equations derived by Valkó and Lopata[214].

A chromatographic polarity parameter ($I - bMR$) has been reported by Kaliszan and Hoeltje[190] where MR is the molar refractivity of the solute examined and b is the stationary phase polarizability coefficient.

The coefficient b was determined by the examination of retention of two stationary phases of different polar character.

Zhang and Shi[215] have evaluated the MOSCED (modified separation of cohesive energy distances) and UNIFAC, method of Fredenslund *et al.*[216] for the prediction of retention indices by a consideration of activity coefficients at infinite dilution for a variety of organic compounds. On non-polar stationary phases with aliphatic and aromatic hydrocarbons the MOSCED predictions were found to be reasonably accurate and markedly better than those given by the UNIFAC procedure.

Relationships between both gas and liquid chromatographic retention parameters and the molecular surface area of polyhalogenated biphenyls has been reported by Hoefler *et al.*[217]. Generally the substitution of hydrogen by halogen atoms (chlorine, bromine, iodine) increased both the retention index and the calculated surface area. On the other hand, fluorine substitution led to a slight increase in molecular surface area, but a reduction in retention.

11. TABULATION OF RECENTLY REPORTED RETENTION INDEX DATA

Many or most reports concerning the retention index relate to small numbers of compounds often for some analytical purpose. These are not included in Table 2 which largely shows compilations of data of larger numbers of compounds.

12. CONCLUSIONS

The retention index has continued to find ever increasing acceptance, despite its shortcomings, particularly with polar stationary phases and where the samples and standards are of widely differing chemical classes. The problems have been known for decades but no more satisfactory general series has been introduced or is probably likely to be introduced. With the widespread use of data processing equipment the interrelation of retention systems and calibration standards is common place and leads to minimisation of some of the difficulties.

The general statement is certainly very useful that the reproducibility of retention indices based on *n*-alkane is good to excellent if the surface tension of the stationary phase is low. This is a characteristic of many non-polar stationary phases, such as hydrocarbons, methyl polysiloxanes, and in such cases retention is only due to absorption. Polar phases often behave very differently. Adsorption at the surface of the moderately polar phase, *i.e.*, polyethylene glycol[362] has been studied and it has been shown that retention data are reproducible if the specific surface area of the liquid phase is the same.

The first 25 years of the retention index scheme was exhaustively reviewed[11] to include most mentions of the retention index, and provided some 1500 reports from the literature equivalent to sixty per annum. The increasing usage of the scheme is

TABLE 2

RECENTLY REPORTED RETENTION INDEX DATA

Compounds		*Ref.*	*Note*[a]
Alkanes	C_4–C_{10}	218	a
	C_3–C_{13}, *n*-	183	a
	C_6–C_{14}, iso-	219	f
	67 C_8–C_{14}	220	a
	31 miscellaneous	221	
	63 C_6–C_9, branched	222	a
	C_3–C_7 alkylcyclopentanes	223	
	C_2–C_6 alkylcyclohexanes	223	
	Cycloheptanes	189	a
	C_{17}–C_{24}, miscellaneous	224	
	20 cyclo-	221	
	Alkenyl cyclo-	363	
	Chlorinated cyclo-	225	
	Bicyclo[4.4.0]decane stereoisomers	226, 227	
Alkenes	71 C_3–C_{13}, *n*-	220, 228	
	47 C_5–C_8	198, 229, 230	
	Cyclo	231	c
	Mono and cyclo-	232	
	Substituted cyclo-	233	
	Alkyl dicyclopentadienes	234	
	C_3–C_7 alkylcyclopentanes	223	
Alkynes	Cyclo	231	c
	Miscellaneous	198	
Hydrocarbons	C_5 petroleum	235, 236	g
	C_6	188	a
	51 hydrocarbons	97	g
	33 hydrocarbons	237	
	100 hydrocarbons	238	g
	200 hydrocarbons	239	
	Coal hydrogenate	240	d, f
	C_4–C_{10}, miscellaneous	241	
	105 C_9–C_{16}, b.p. 150–175°C	242	
	Nitrated polynuclear	243	e
	200 petroleum	244	
	43, b.p. 50–70°C	245	
	200, b.p. 0–200°C	246	
	Chlorinated C_1–C_3	247	
	26 diterpenic	248	
Aromatic	Methylbenzenes	249	
	34 alkyl	250	a
	Various alkyl	251, 252	a
	30 alkyl	253	
	Miscellaneous alkyl	186	a
	C_2–C_6 alkyl	254	
	13 alkenyl	255	a
	Monosubstituted alkyl	256	
	C_6–C_{10} monocyclic	257	
	10 polycyclic	205, 258	
	45 polycyclic	208	a

TABLE 2 (*continued*)

Compounds		*Ref.*	*Note*[a]
	polyaromatic	259, 260	
	C_{14}–C_{17}, alkyl	261	
	C_6–C_{12}, alkyl	262	f
	57 C_6–C_{14}, monocyclic	263	f
	25 C_6–C_{14}, bicyclic	263	f
	Mononitrated poly	209	
	56 nitrated	264, 265	
	Poly S- and N-heterocyclic	77	k
	Hydroxy	266	
	Methoxy	266	
	Chlorinated	266	
	Chlorinated	267	c
	Chlorinated	180	a
	51 complex	268	
	Benzene and chloro- and nitro derivatives	269	d
	Chloranisoles	270	d
	19 chloranisoles	271	
	o-Methoxyanisoles	270	d
	Isomeric chlorophenols	272	c
	Halogenated benzenes and anisoles	273	
	9 chlorinated 4-hydroxybenzaldehydes	274	
	9 chlorinated veratroles	275	
	2,5-Dichloroterephthalic acid derivatives	193	a
	Pyridines, alkyl	276	
	Pyridines, methyl	277	
	28 alkyl quinolines	278	a
	25 alkyl quinolines	279	
	24 alkyl quinoline bases	280	
	Monosubstituted pyrazines	281	a
	100 pyrazines	282	
	Alkyl diphenyls	283	c
	27 chlorinated biphenyls	284	
	57 halogenated biphenyl	217	
	7 *p*-quinones	285	
	Alkyl naphthalenes	286	
	12 Monomethylbenz[a]anthracenes	201	c
	Chlorinated dibenzo-*p*-dioxins	287	a
	9-substituted carbazoles	211	a
Drugs	Miscellaneous	288	
	275 drugs	289	
	61 drugs	290	
	101 urinary organic acids	291	
	8 basic and neutral	292	h
	Neuroleptic	293	
	175 basic	294	
	Acidic, neutral and basic	295, 296	
	16 antirheumatic	297	
	100 poisonous compounds	298	
	61 volatile compounds in blood	290	
	33 benzodiazepine drugs	299	

(*Continued on p. 118*)

TABLE 2 (*continued*)

Compounds		*Ref.*	*Note*[a]
Esters	C_1–C_{16} *n*-alkyl C_3–C_5 isoalkyl chloroacetates	300	
	C_1–C_{18} *n*-alkyl butanoates and chlorinated derivatives	301	c
	C_1–C_{18} alkyl and 2-chloro and 3-chloropropanoates	302	c
	2-, 2,2-, 2,2,2-chloroesters of C_2–C_{20} n-alkanoates	303	c
	C_1–C_{12} *n*-alkyl benzoates and pentafluorobenzoates	304	c
	C_1–C_{18} alkyl 2- and 3-chloroacetates	303	c
	C_1–C_{18} *n*-alkyl chloro, dichloro, trichloracetates	305	c
	Chlorinated methyl propanoates and butanoates	306	
	Benzoyl and monochlorobenzyl esters of unsaturated acids	307, 308	
	Unsaturated benzoic and monochlorinated esters	309	
	Benzoyl and pentafluorobenzyl esters	310	
	Ethyl and chloroethyl monochlorobenzoates	311	c
	C_1–C_{12} *n*-alkyl benzoates, 4-nitro and 3,5-dinitro derivatives	312	
	Acyl and halogenated acyl esters of unsaturated alcohols	313	
	Branched C_3–C_5 alkyl halogenated acetates	314	
	C_1–C_{10} alkyl, C_2–C_{10} alkanoates and haloalkyl derivatives	315	
	35 aliphatic esters	250	
	173 aliphatic saturated and unsaturated esters	316	
	Methyl esters of aryl aliphatic acids	317	
	C_1–C_6 *n*-, C_3–C_6 isoalkyl acrylates	318	
	C_1–C_{18} alkyl acrylates and methacrylates	319	
	Polyoxyethylene glycol diacetates	320	j
	Chloropropyl esters of aliphatic acids	321	
	Haloalkylsalicylic esters	322	
	Chlorinated phenyl acetates	323	
	9 methyl esters of resin acids	324	
	Orthophosphate esters	325	
	Barbiturate esters	326	
	Glycidic esters	327	
Miscellaneous	600 pesticides	328	b
	22 chemical warfare agents	329	h
	37 sulphur vesicants	330	h
	12 nitrogenous siloxanes	185	a
	Alkylalkoxysilanes	331	
	Silazanes and siloxazines	332	
	Trimethylsilyl amino acids	333	
	Epoxides C_4–C_5	334	
	Racemic and enantiomeric amino acids	335	f
	10 thiols	336	
	Alkyl disulphides	337	
	18 dialkyl sulphides	336	
	C_1–C_2 alkylated benzothiophenes	338	
	Substituted dibenzo[*b*,*f*]thiepins	339	c
	Constituents 14 essential oils	340	
	56 essential oil constituents	341	
	50 organic micropollutants	342	
	4 organotin compounds	343	
	Organophosphorus compounds	344	
	16 C_6 alkanols	345	
	Saturated alcohols	178	a
	Polycyclic alcohols	346	a
	2-Nitro-1-alkanols	347	

TABLE 2 (*continued*)

Compounds	*Ref.*	*Note*[a]
o-Methyl inositols	348	
Oxygenated compounds	349	
Alkyl ketones	350	h
Polyoxyethylene derivatives	351	d
Polyoxyethylene surfactants	352	j
28 heterocyclic nitrogen compounds	210	a
68 monoalkylnitrogen heterocyclic compounds	210	a
15 oxygen-containing adamantanes	353	f
Secondary aliphatic amines	354	a
Secondary amines	355	a
Aliphatic and heterocyclic amines	356	
Formamidines, N,N′-dialkyl	357	
Formamidines, N,N′-dimethyl	358	
207 dimethylbenzamidines and benzylidene amines	359	
Alkyl porphyrins	360	
7 triochothecenes	361	

[a] Notes: a = correlations with physico-chemical properties; b = emergence temperatures; c = $I+$ retention increments; d = retention increments; e = I and PAH indices; f = I and temperature dependence; g = unified retention indices; h = programmed indices; i = electronic interaction indices; j = arithmetic retention indices; k = Lee indices.

indicated in this —a very selective— review where the majority of works containing data of a few compounds have been omitted; nevertheless the bibliography is proportionately much greater.

13. ACKNOWLEDGEMENTS

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14. SUMMARY

The development of the retention index over 30 years is summarised and advances that have occurred in recent years are highlighted.

The work follows an exhaustive review published by Budahegyi *et al.* in 1983, but is much more selective and does not include the many reports of isolated retention indices.

The review includes alternative logarithmic index schemes and other reference series that have been reported and which were not included in the earlier work. The work concludes with a tabulation of recently available compilations of retention indices.

15. NOTE ADDED IN PROOF

This article is based upon a paper presented at the *17th International Symposium on Chromatography* held in Vienna, 25–30 September 1988. From the onset it was the authors' intention to present a selective account of recent developments in Kovats' retention index and related retention scales. In this respect it may be regarded as being complementary to the accompanying article by Dr. Takács and his co-workers (this issue, pp. 1–92). Together we believe that the combined articles will represent the most comprehensive and authoritative account of Kovats' retention index scheme to have been published to date.

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INTERACTIVE RETENTION INDEX DATABASE FOR COMPOUND IDENTIFICATION IN TEMPERATURE-PROGRAMMED CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

A procedure is described and evaluated that allows the calculation of linear temperature-programmed retention indices from accurate Kováts retention indices on a given stationary phase and their temperature variation coefficients. The influence of experimental factors such as column film thickness, phase ratio and variation of Kováts retention indices, column dead time and carrier gas flow-rates are examined. The calculation accuracies are ≤ 0.5 retention index units in most instances. The applicability and limitations of the procedure are discussed.

INTRODUCTION

Large numbers of isothermal gas chromatographic retention data have been published as Kováts retention indices. The use of Kováts retention indices has a great advantage over relative retention times, retention volumes or capacity ratios in that they are independent of the film thickness, column dimensions and phase ratio. Owing to the advances in column technology, highly reproducible (expressed as Kováts' indices) columns are commercially available for non-polar and some polar stationary phases.

The prediction or calculation of linear temperature-programmed retention indices (LTPRI) from isothermal data has been explored in the past[1–6] and different approaches were proposed and evaluated. Owing to the difference between the LTPRI and Kováts systems in the definition of retention index, the correct way to convert isothermal retention data to LTPRI is through the thermodynamic parameters of components as established by Curvers *et al.*[1]. It is feasible in theory to transfer the isothermal data (capacity ratios or Kováts retention indices) in terms of entropy and enthalpy of each component from one column to another for columns with the same stationary phase but different dimensions. However, in practice, this is not as easy as it may seem. The entropy term $[\exp(\Delta S/R)/\beta]$ is dependent on the phase ratio (β) and is difficult to determine accurately owing to the uncertainty in the measurement of the phase ratio. It has been observed experimentally that the enthalpy terms are also

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dependent on the phase ratio[1]. Some of them are unexpectedly large, whereas theoretically they should remain constant.

The objectives of the study reported here were (a) direct utilization of Kováts retention indices, either measured or published, for calculating LTPRI; and (b) to find a means of eliminating the necessity to determine the film thickness or phase ratio and the corresponding changes in entropy and enthalpy.

THEORY

Calculation

For a given component i at a given temperature T, the Kováts retention index is

$$I(i) = 100z + 100 \cdot \frac{\log t'_R(i) - \log t'_R(z)}{\log t'_R(z+1) - \log t'_R(z)} \tag{1}$$

Substitution of $t'_R(i)/t'_R(j) = k(i)/k(j)$ in eqn. 1 and rearranging yields

$$\log k(i) = \frac{I(i) - 100z}{100} \cdot \log\left[\frac{k(z+1)}{k(z)}\right] + \log k(z) \tag{2}$$

Converting both sides of the equation into natural logarithm gives

$$\ln k(i) = \frac{I(i) - 100z}{100} \cdot \ln\left[\frac{k(z+1)}{k(z)}\right] + \ln k(z) \tag{3}$$

One should bear in mind that all variables in eqns. 1–3 are temperature dependent. To make it explicit, $k(i,T)$ is used instead of $k(i)$ in the following equations.

Using the well known equation

$$\ln k(i,T) = \ln\left[\frac{\alpha(i)}{\beta}\right] - \frac{\Delta H(i)}{RT} \tag{4}$$

where $\alpha = \exp[\Delta S(i)/R]$, together with eqn. 3, we obtain the desired equations:

$$-\frac{\Delta H(i)}{R} = \left[\ln k(i,T_1) - \ln k(i,T_2)\right]\left(\frac{T_1 T_2}{T_2 - T_1}\right) \tag{5}$$

and

$$\frac{\alpha(i)}{\beta} = \exp\left[\ln k(i,T) - \frac{\Delta H(i)}{RT}\right] \tag{6}$$

where T can be T_1 or T_2.

Apart from the Kováts retention index of components at two temperatures, the only additional information needed for the calculation of the entropy and enthalpy terms is the adjusted retention time (or capacity ratio) of n-alkanes measured at two

corresponding temperatures on the column used for analysis. Subsequent calculations of the temperature-programmed retention temperature or retention time of each component use the well defined relationship[1]

$$\int_{T_0}^{T_R} \frac{dT}{t_0(T)\left[1 + \frac{a}{\beta}\exp\left(\frac{-\Delta H}{RT}\right)\right]} = r \tag{7}$$

Van den Dool and Kratz's[7] definition of the LTPRI is used in the final calculation.

EXPERIMENTAL

A Model 5790A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with an HP 7672A auto-sampler was used for column testing. The instrument was provided with a two-point temperature calibration at 130.0 and 320.0°C. The temperature control was better than 0.1°C. An external high-precision pressure gauge was installed to allow pressure readings of 0.001 bar. An HP Model 3388 computer integrator was used for data handling.

A Mega Series 5300 gas chromatograph (Carlo Erba, Milan, Italy) was used for testing squalane columns. An external quartz thermometer (Type 2801, Hewlett-Packard) was used to correct the oven temperature under isothermal operation. A Spectra-Physics (Santa Clara, CA, U.S.A.) SP4290 integrator was used for data handling.

Four different cross-linked columns were randomly selected from the Ultra Performance series of Hewlett-Packard and two from Chrompack (Middelburg, The Netherlands). Their characteristics are given in Table I.

Helium was used as the carrier gas. The septum-flush flow-rate was 15 ml/min; the splitting ratio was set at 1:100 for the HP 5790A and 1:200 for the Mega Series 5300 instruments.The temperatures of the injection port and detector block were set at 250°C for the HP 5790A and 200°C for the Mega Series 5300 instruments.

Samples containing hydrocarbons, aromatics, ketones, esters and alcohols were used with both non-polar and weakly polar columns. AT-class microcomputers with math coprocessors were used for all computations. Software was written in Quick-Basic (Micro-Soft).

TABLE I

COLUMN CHARACTERISTICS

Column No.	*Stationary phase*	*Length (m)*	*I.D. (mm)*	*Film thickness (μm)*	*Manufacturer*
1	Squalane	50	0.22	0.21	Chrompack
2	OV-1	25	0.31	0.52	Hewlett-Packard
3	SE-54	25	0.31	0.52	Hewlett-Packard
4	OV-1	20	0.32	1.2	Chrompack
5	OV-1	25	0.31	0.52	Hewlett-Packard
6	SE-54	25	0.31	0.52	Hewlett-Packard

RESULTS AND DISCUSSION

The reliability of the calculated LTPRI is influenced by several factors, *viz.*, $\Delta H/RT$, $\Delta S/R$, β, $t_0(T)$ and the accuracy of the isothermal data used for the calculation; for clarity, we discuss them separately.

Temperature dependence of entropy and enthalpy terms

As both the entropy and enthalpy terms (*cf.*, eqns. 5 and 6) are temperature dependent, their values determined according to the procedure used in refs. 1–3 and the approach proposed in this paper are actually average values between the two temperatures of the isothermal measurements. The values of both terms depend not only on the temperatures of the isothermal measurements, but also on the difference between the two temperatures. When the elution temperature of a component is much higher than the upper isothermal measurement temperature, the term $\alpha/\beta\exp[-\Delta H/(RT)]$ will be smaller than it should be (*cf.*, data in ref. 1, Table 3), and the calculated T_r will be lower than the experimental value. Therefore, the optimum temperatures for measuring the isothermal retention indices should be selected as discussed by Curvers *et al.*[1], in order to obtain a better fit with the calculated and experimental LTPRIs.

The influence of the selected isothermal temperatures on the calculated LTPRI is different in both concepts. The retention temperatures of components and the adjacent *n*-alkanes are calculated using entropy and enthalpy terms obtained under identical conditions. In our approach, this is accomplished by using (a) the Kováts retention indices at two isothermal temperatures (which are known to have very good column-to-column reproducibility) and (b) the adjusted retention times of *n*-alkanes at two corresponding isothermal temperatures measured on the column to be used.

Systematic errors are partially cancelled in the calculation of LTPRI, because the shifts of the calculated T_r for a given component and the adjacent *n*-alkanes are in the same direction and of a similar magnitude. A comparison of calculated and measured LTPRIs is presented in Tables II–V for four columns differing either in stationary phase or film thickness, with different programming rates. The differences between the measured and calculated retention indices are within 0.5 retention index units (i.u.) in all instances, except for 1-butanol, for which there is a non-linear relationship between $\ln k$ and $1/T$. Note that the elution temperatures of some components (in the bottom parts of Tables III and IV at a programming rate of 8°C/min) are much higher than the temperatures in the isothermal measurements, and these do not cause an additional error in the calculated LTPRI. Unlike in the former approach[1], the choice of temperature in isothermal measurements is not so critical with the procedure presented here. Considering both the analysis time and the accuracy of the measurement, a capacity ratio (k') between 0.5 and 30 should be satisfactory. The temperature difference between the two measurements should be as large as possible.

Effect of film thickness and phase ratio

The entropy and enthalpy terms of some components on columns 2 and 4 are given in Table VI. The values were calculated using tabulated Kováts retention indices measured on column 2 at two temperatures and the adjusted retention times of *n*-alkanes obtained from two isothermal runs for each column. The agreement of the

TABLE II

COMPARISON OF CALCULATED AND MEASURED LTPRI VALUES ON COLUMN 1 (SQUALANE)

T_0 = 40.0°C.

Component	*Isothermal (°C)*		*Programming rate (°C/min)*			
	50.0	*70.0*	*1.5*		*2.5*	
			$I_{calc.}$[a]	ΔI[a]	$I_{calc.}$[a]	ΔI[a]
2,2-Dimethylpentane	625.6	626.9	617.8	−0.1	618.6	−0.1
2,4-Dimethylpentane	629.8	630.5	621.4	0.0	622.2	0.0
Benzene	637.2	641.8	626.7	−0.3	628.1	−0.3
3,3-Dimethylpentane	658.9	661.5	648.0	0.0	649.5	0.1
2-Methylhexane	666.6	666.9	657.0	−0.2	658.1	−0.1
1-*cis*, 3-Dimethylcyclopentane	682.7	686.0	675.6	0.3	676.9	0.3
3-Ethylpentane	686.0	687.1	680.4	0.0	681.2	−0.1
2,5-Dimethylhexane	728.4	729.0	722.5	−0.1	723.7	−0.1
2,4-Dimethylhexane	731.9	732.9	725.6	−0.1	727.0	0.0
Ethylcyclopentane	733.8	737.5	727.0	0.3	728.9	0.2
2,3-Dimethylhexane	760.1	761.5	753.1	0.2	754.9	0.3
3,4-Dimethylhexane	770.6	772.6	764.6	0.1	766.4	0.0
1-*cis*, 3-Dimethylcyclohexane	785.0	789.8	781.5	0.3	783.8	0.4
1-*trans*, 2-Dimethylcyclohexane	801.8	807.5	802.0	0.3	803.5	0.4
1-*trans*, 3-Dimethylcyclohexane	805.6	810.8	805.3	0.4	806.8	0.4
Ethylbenzene	834.6	839.8	832.4	0.4	835.1	0.5
Isopropylcyclopentane	812.1	817.0	811.1	0.3	812.9	0.4
1,4-Dimethylbenzene	849.1	854.0	847.0	0.3	849.9	0.5

Component	*Isothermal (°C)*		*Programming rate (°C/min)*					
	50.0	*70.0*	*1.0*		*2.0*		*3.0*	
			$I_{calc.}$[a]	ΔI[a]	$I_{calc.}$[a]	ΔI[a]	$I_{calc.}$[a]	ΔI[a]
2-Methyl-2-butene	514.3	514.4	508.9	−0.1	509.1	−0.1	509.2	−0.2
4-Methyl-1-pentene	549.4	550.4	536.8	0.0	537.5	0.0	538.0	−0.1
2,3-Dimethylbutane	567.3	568.8	555.2	−0.1	556.0	0.0	556.6	−0.1
2-Methyl-1-pentene	580.1	580.5	571.3	−0.1	571.8	−0.1	572.3	−0.1
Methylcyclopentane	627.9	630.9	618.7	0.2	619.9	0.3	620.8	0.3
2,4-Dimethylpentane	629.8	630.5	620.9	−0.1	621.9	−0.1	622.6	−0.1
Benzene	637.2	641.8	625.9	−0.4	627.5	−0.3	628.7	−0.3
Cyclohexane	662.7	667.1	650.6	0.3	652.6	0.4	654.2	0.5
2-Methylhexane	666.6	666.9	656.4	−0.1	657.7	−0.1	658.7	0.0
1-Heptene	681.8	682.3	674.6	−0.2	675.7	−0.1	676.4	−0.1

[a] In all tables, ΔI = LTPRI(calculated) − LTPRI(measured).

Kováts retention indices measured on both columns (*cf.*, Tables III and V) is within 0.3 i.u. for the test sample, although the columns have a different film thickness or phase ratio and are from different manufacturers.

The ratio β(column 2)/β(column 4) was found to be 2.3 from the specification of

TABLE III

COMPARISON OF CALCULATED AND MEASURED LTPRI VALUES ON COLUMN 2

T_0 = 40.0°C; P_i = 0.600 kg/cm^2; u = 34 cm/s. Flow-rates measured at 60°C here and in all following tables.

Component	*Isothermal (°C)*				*Programming rate (°C/min)*						*Elution temperature (°C)*
	45.0	*60.0*	*65.0*	*75.0*	*2.0*		*4.0*		*8.0*		
					$I_{calc.}$	ΔI	$I_{calc.}$	ΔI	$I_{calc.}$	ΔI	
2-Methyl-2-butene	520.0		519.8		513.6	−0.1	513.8	0.0	514.2	−0.1	
4-Methyl-1-pentene	556.7		557.1		545.6	0.1	546.2	0.1	547.0	0.0	
3-Dimethylbutane	566.3		567.7		555.8	0.1	556.4	0.1	557.3	−0.2	
2-Methyl-1-pentene	588.0		588.0		582.7	−0.2	583.0	−0.2	583.5	−0.1	
2,2-Dimethylpentane	624.8		628.1		617.6	−0.1	618.3	−0.1	619.4	0.0	
Methylcyclopentane	628.2		631.1		620.1	−0.1	621.1	−0.1	622.6	−0.4	
2,4-Dimethylpentane	630.5		631.3		622.2	0.0	623.0	−0.2	624.2	0.1	
Benzene	651.4		655.8		640.7	−0.2	642.3	−0.1	644.5	−0.1	
3,3-Dimethylpentane	656.2		659.0		645.8	−0.1	647.2	0.0	649.2	0.0	
Cyclohexane	660.6		665.6		650.1	−0.2	651.9	−0.1	654.4	−0.1	
2-Methylhexane	667.6		668.1		658.4	−0.1	659.5	−0.1	660.9	−0.1	
2,3-Dimethylpentane	669.9		672.0		660.8	0.0	662.0	0.0	663.9	0.2	
1,1-Dimethylcyclopentane	673.3		677.4		664.5	−0.1	666.0	0.0	668.3	0.1	
1-*cis*, 3-Dimethylcyclopentane	683.3		687.5		676.8	−0.2	678.2	0.0	680.3	0.4	

3-Ethylpentane	686.4		687.5		681.2	0.0	682.0	0.1	683.1	0.1	
1-Heptene	688.6		688.7		684.2	−0.1	684.7	−0.1	685.4	−0.2	
1-*cis*, 2-Dimethylcyclopentane	719.5		723.8		714.4	−0.1	715.8	−0.1			
2,2-Dimethylhexane	722.1		723.8		716.6	−0.1	717.8	0.0			
3,3-Dimethylhexane	741.2		744.0		733.2	−0.1	735.2	−0.1	737.8	0.2	
Toluene	753.6		758.4		745.3	−0.1	747.9	−0.2	751.3	0.1	
2,3-Dimethylhexane	760.3		762.2		752.3	0.0	754.4	−0.1	756.9	0.2	
3,4-Dimethylhexane	769.0		771.1		761.9	0.0	764.0	−0.1	766.7	0.2	
1-*cis*, 3-Dimethylcyclohexane	774.9		780.3		768.7	0.0	771.4	−0.1	774.9	0.2	
1-*cis*, 2-Dimethylcyclohexane	822.2		828.8		819.0	−0.1	822.0	−0.1	825.8	0.3	
n-Propylcyclopentane	827.2		831.6		823.3	−0.1	825.9	−0.1	829.0	0.2	
Ethylbenzene	844.6		849.7		840.0	−0.1	843.4	−0.1	847.2	0.2	
1,4-Dimethylbenzene	854.2		859.0		849.6	−0.2	853.1	0.0	856.9	0.3	
1.2-Dimethylbenzene	874.0		879.6		871.4	−0.1	874.9	−0.1	879.0	0.3	
Dichloromethane		516.9		516.5	511.6	0.1	511.7	0.0	512.0	0.2	52.62
1-Butanol		646.8		643.9	639.9	−0.7	640.4	−0.5	641.1	−0.3	58.83
Toluene		756.6		759.9	745.3	0.0	747.8	0.0	751.0	0.0	69.10
n-Hexyl chloride		842.9		844.6	836.3	−0.1	838.7	0.0	841.2	0.1	81.12
3-Heptanone		866.2		866.5	861.2	−0.2	863.1	0.1	864.6	0.0	84.67
p-Chlorotoluene		936.3		941.3	932.0	0.1	935.9	0.2	940.6	0.2	96.69
1-Heptanol		954.9		952.8	952.9	−0.1	953.1	0.1	952.4	−0.2	98.62
1,2,4-Trimethylbenzene		976.2		980.5	973.7	0.1	977.5	0.3	981.6	0.3	103.41

TABLE IV

COMPARISON OF CALCULATED AND MEASURED LTPRI VALUES ON COLUMN 3 (SE-54)

T_0 = 40.0°C; P_i = 0.600 kg/cm^2; u = 36 cm/s.

Component	*Isothermal (°C)*				*Programming rate (°C/min)*						*Elution temperature (°C)*
	45.0	*60.0*	*65.0*	*75.0*	*2.0*		*4.0*		*8.0*		
					$I_{calc.}$	ΔI	$I_{calc.}$	ΔI	$I_{calc.}$	ΔI	
2-Methyl-2-butene	522.9		523.0		515.5	0.0	515.7	0.0	516.2	0.1	
4-Methyl-1-pentene	558.7		559.2		547.4	0.0	547.8	−0.1	548.7	−0.2	
2,3-Dimethylbutane	566.2		567.8		555.3	−0.1	555.9	−0.2	556.9	−0.3	
2-Methyl-1-pentene	590.7		591.2		586.3	−0.2	586.6	−0.2	587.0	−0.2	
2,4-Dimethylpentane	629.3		629.8		621.1	−0.1	621.8	−0.1	622.8	−0.3	
Methylcyclopentane	632.3		635.8		623.3	0.0	624.2	−0.2	625.8	−0.1	
Benzene	666.5		667.4		656.3	−0.1	657.8	−0.2	660.1	0.0	
1-Heptene	691.8		691.8		688.3	−0.1	688.7	−0.1	689.2	−0.1	
Dichloromethane		537.8		537.9	527.5	0.0	527.8	0.0	528.4	0.1	52.36
1-Butanol		661.9		959.6	654.6	−1.2	655.2	0.8	655.9	−0.6	58.50
Toluene		771.9		775.4	760.9	0.0	763.4	0.0	766.6	0.0	68.98
n-Hexyl chloride		858.2		859.7	851.4	−0.1	853.9	0.1	856.3	0.0	81.14
3-Heptanone		887.5		887.4	885.1	−0.2	885.9	0.0	886.5	0.0	85.65
p-Chlorotoluene		956.4		961.7	951.9	0.1	956.3	0.3	961.3	0.3	97.69
1-Heptanol		971.1		969.2	969.2	−0.1	969.4	0.2	968.8	−0.2	98.90
1,2,4-Trimethylbenzene		991.9		996.6	990.6	0.0	994.0	0.2	998.1	0.2	103.71

TABLE V

COMPARISON OF CALCULATED AND MEASURED LTPRI VALUES ON COLUMN 4

T_0 = 40.0°C.

Component	*Isothermal (°C)*		*Programming rate (°C/min)*					
	45.0	*65.0*	*2.0*		*4.0*		*8.0*	
			$I_{calc.}$	ΔI	$I_{calc.}$	ΔI	$I_{calc.}$	ΔI
2-Methyl-2-butene	520.0	519.8	513.7	0.0	514.1	0.1	514.7	0.1
4-Methyl-1-pentene	556.7	557.1	546.0	0.2	546.9	0.3	548.2	0.4
2,3-Dimethylbutane	566.3	567.7	556.2	0.2	557.1	0.2	558.6	0.3
2-Methyl-1-pentene	588.0	588.0	583.0	0.1	583.5	−0.1	584.1	0.0
MethylCyclopentane	628.2	631.3	620.9	0.1	622.3	0.1		
2,4-Dimethylpentane	630.4	631.3	623.0	0.0	624.2	0.0		
Benzene	651.3	655.4	642.0	0.2	644.2	0.5	647.2	0.6
Cyclohexane	660.6	665.6	651.6	0.2	654.0	0.4	657.3	0.6
2-Methylhexane	667.6	667.7	659.5	0.1	661.0	0.0	662.8	0.0
1-Heptene	688.6	688.7	684.7	0.0	685.5	0.0	686.4	0.0

the columns. However, the ratio of the entropy terms of a component [(α/β) (column 4]/[(α/β) (column 2)] between the two columns is larger than 4 (*cf.*, Table VI). The deviation from theory could be the result of inaccurate values of the phase ratio or the strong surface effect on the stationary phase. The latter causes the properties of a coated stationary phase to deviate from that of the bulk system, and the entropy and enthalpy of a component to depend on the film thickness. In either instance, it is possible to correct this ratio based on the entropy terms of a known component measured on both columns, without the need to know the absolute values of the phase ratios or the degree of the surface effect. With this correction, the Curvers *et al.* concept[1,2] is still valid if the enthalpy terms are constant.

TABLE VI

CALCULATED ENTROPY AND ENTHALPY TERMS ON COLUMNS 2 and 4

Component	$\alpha/\beta(\times 10^{-7})$		$-\Delta H/R$ (K)	
	Column 2	*Column 4*	*Column 2*	*Column 4*
2-Methyl-2-butene	398.25	1624.4	2890.7	2753.8
4-Methyl-1-pentene	339.86	1377.4	3048.1	2913.6
2,3-Dimethylbutane	367.54	1487.4	3051.1	2917.2
2-Methyl-1-pentene	264.38	1065.3	3219.1	3086.8
n-Hexane	242.05	973.22	3282.3	3150.8
Methylcyclopentane	303.87	1231.8	3290.8	3156.5
Benzene	288.90	1179.1	3373.4	3236.8
Cyclohexane	305.44	1250.0	3382.3	3244.8
2-Methylhexane	149.81	614.36	3629.1	3490.9
2,4-Dimethylpentane	219.03	888.52	3401.5	3267.0
1-Heptene	127.33	525.39	3741.1	3600.8
n-Heptane	115.96	480.10	3803.6	3662.2

The data in Table VI showed that the enthalpy terms on the two columns were not consistent. Their differences are about 4% on average, which alone could generate at least a 30 i.u. difference in calculated LTPRIs if the enthalpy terms are taken as the basic data. The film thickness dependence of the enthalpy term invalidates the attempt to transfer the entropy and enthalpy terms from one column to another as proposed by Curvers *et al.*[1].

The procedure described here corrects the variations in film thickness, phase ratio or the surface effect of the phase through measurements on *n*-alkanes under isothermal conditions, without the need to know them. The retention times of *n*-alkanes together with the Kováts retention indices are used to calculate the entropy and enthalpy terms of each component on the column to be used for analysis. It is as if all the components were measured on this column with this procedure, when the reproducibility of Kováts retention indices of different columns can be guaranteed. Note that some components, which have larger dI/dT values (*e.g.*, benzene and cyclohexane; *cf.*, Table V) show slightly larger deviations between the calculated and mean real LTPRIs at higher programming rates for column 4. The probable reason is that the average programming rate is smaller than expected, owing to bundling of the column coils. For all other columns the coils were regularly distributed in single layers on a light metal frame. The coils at the inner side of the bundle cannot follow the set programming rate. Obviously this deviation of the actual column temperature from the set value will have a larger effect on components with a high dI/dT.

Effect of variation of Kováts retention indices

As isothermal retention indices are used as the basic data for the calculation of LTPRIs, their variation between different columns will certainly have an influence on the LTPRIs. We studied this effect by varying the Kováts retention indices by ± 0.5 i.u., which is an acceptable simulation of the real situation. The results in Table VII show that the error of calculated LTPRIs and the deviation of the Kováts retention indices used for the calculation are of the same order of magnitude or even less.

Effect of variation of column dead time

The column dead time reflects the integrated effects of column length and flow-rate. It has to be taken into account in the calculation of the elution temperature, T_r, in temperature-programmed gas chromatography (*cf.*, eqn. 7).

The column dead time, t_0, is cancelled in the calculation of LTPRI when measured data are used. In the calculation of T_r, however, such a process is simulated, and the dead time $t_0(T)$ will certainly affect the T_r value. As the same dead time $t_0(T)$ is used for the calculation of the elution temperature of the components for a given programmed run, systematic errors in $t_0(T)$ can also be partly cancelled in the calculation of LTPRIs. From this point of view, some variation of $t_0(T)$ is acceptable. The t_0 values under isothermal conditions are involved in the calculation of the adjusted retention times or capacity ratios of *n*-alkanes, and have a direct influence on the values of the entropy and enthalpy terms.

In this study, methane was used as the dead-time marker, not because it can give the best accuracy in t_0 measurements, but because most of the published data were measured using the retention time of methane as t_0. The consistency in choosing the marker will improve the accuracy of the calculated LTPRIs, particularly for earlier

TABLE VII

EFFECT OF VARIATION OF ISOTHERMAL RETENTION INDEX ON LTPRI

Calculated for Column 2. T_0 = 40.0°C; r = 8.0°C/min.

Compound	*Isothermal (°C)*		$I_{calc.}$
	60.0	*75.0*	
Dichloromethane	516.90	516.50	512.0
Variation	−0.50	+0.50	−1.0
	−0.50	+0.0	+0.3
	+0.0	+0.50	−0.3
	+0.0	−0.50	+0.3
	+0.50	+0.0	+0.7
	+0.50	+0.50	+0.4
Toluene	756.62	759.90	751.0
Variation	−0.50	+0.50	−0.9
	−0.50	+0.0	−0.7
	+0.0	−0.50	+0.1
	+0.0	+0.50	+0.2
	+0.50	+0.0	+0.6
	+0.50	−0.50	+0.9
	+0.50	+0.50	+0.5
p-Chlorotoluene	936.25	941.30	940.6
Variation	−0.50	+0.50	+0.4
	−0.50	+0.0	−0.1
	+0.0	+0.50	+0.5
	+0.0	−0.5	−0.5
	+0.50	+0.0	+0.1
	+0.50	−0.50	−0.4

eluting components. This is illustrated in Table VIII, where LTPRIs were calculated using measured and extrapolated dead times on two different columns. For components having a retention index above 600 no significant differences were observed for the two methods.

Influence of carrier flow-rate or inlet pressure

For well deactivated columns and a sample amount far below the maximum capacity of the column, the entropy and enthalpy terms are expected to be independent of carrier gas flow-rate or inlet pressure under normal operating conditions, particularly when helium is used as the carrier gas and the inlet pressure is lower than 2 bar. For unknown reasons, the entropy and enthalpy terms are also affected by the flow-rate or pressure drop, as shown in Table IX. The data were calculated using measured Kováts retention indices and adjusted retention times of *n*-alkanes at corresponding isothermal temperatures at different inlet pressures. The effects of about a 1.5% variation in the entropy and enthalpy terms on the calculated LTPRI is shown in Table X. By comparison of these values with the results in Table IX, the error in the calculated LTPRIs caused only by the different flow-rate or inlet pressure can be calculated when the entropy and enthalpy terms are used as the basic data.

TABLE VIII

COMPARISON OF CALCULATED[8] AND MEASURED LTPRI VALUES ON COLUMNS 2 AND 3 USING DIFFERENT t_0 VALUES IN THE CALCULATION

T_0 = 40.0°C; r = 8.0°C/min.

Component	*Column 2*				*Column 3*			
	Calc. with				*Calc. with*			
	Measured t_0	ΔI	*Calc.* t_0	ΔI	*Measured* t_0	ΔI	*Calc.* t_0	ΔI
Dichloromethane	512.0	0.2	512.8	1.0	528.4	0.1	529.5	1.2
4-Methyl-1-pentene	547.0	0.0	548.0	1.0	548.7	−0.2	549.7	0.8
Methylcyclopentane	622.6	−0.4	622.9	−0.1	625.8	−0.3	626.2	0.1
1-Heptene	685.4	−0.2	685.6	0.0	689.2	−0.1	689.4	0.1
Toluene	751.0	0.0	751.2	0.2	766.6	0.0	766.8	0.2
n-Hexyl chloride	841.2	0.1	841.3	0.2	856.3	0.0	856.4	0.1
3-Heptanone	64.6	0.0	864.7	0.1	886.6	0.1	886.6	0.1
p-Chlorotoluene	940.6	0.2	940.7	0.3	961.3	0.3	961.4	0.4
1-Heptanol	952.4	−0.2	952.4	−0.2	968.8	−0.2	968.8	−0.2
1,2,4-Trimethylbenzene	981.6	0.3	981.7	0.4	998.1	0.2	998.1	0.2

These effects are avoided when using the procedure proposed in this paper. The results for both measured and calculated LTPRIs for two different columns at two different flow-rates or inlet pressures are given in Table XI and also Tables III and IV. The error was less than 0.4 i.u. for all the components tested, except the first

TABLE IX

VARIATION OF ENTROPY AND ENTHALPY TERMS WITH CARRIER FLOW-RATE OR INLET PRESSURE

Measured on column 2. $-\Delta H/R$ in Kelvin.

Component	*Inlet pressure (kg/cm²)*							
	0.300		*0.450*		*0.600*		*0.800*	
	$-\Delta H/R$	α/β^a	$-\Delta H/R$	α/β^a	$-\Delta H/R$	α/β^a	$-\Delta H/R$	α/β^a
2-Methyl-2-butene	2884.1	400.80	2867.3	424.96	2890.7	398.25	2923.6	353.52
4-Methyl-1-pentene	3041.9	341.88	3037.1	348.98	3048.1	339.86	3055.0	327.73
2,3-Dimethylbutane	3047.8	366.94	3049.3	366.98	3051.1	367.54	3069.3	342.25
2-Methyl-1-pentene	3212.9	266.55	3217.8	264.11	3219.1	264.38	3225.3	255.71
n-Hexane	3278.9	241.89	3283.4	239.85	3282.3	242.05	3289.8	233.26
Methylcyclopentane	3292.4	299.24	3294.6	298.68	3290.8	303.87	3299.1	292.28
2,4-Dimethylpentane	3400.1	217.56	3402.5	217.14	3401.5	219.03	3408.9	211.29
Benzene	3361.7	296.43	3363.3	296.48	3373.4	288.90	3371.0	287.23
Cyclohexane	3371.6	312.46	3376.4	309.32	3382.3	305.44	3382.7	301.13
2-Methylhexane	3630.5	147.48	3624.3	151.21	3629.1	149.81	3631.7	146.66
1-Heptene	3723.9	132.98	3729.3	131.40	3741.1	127.33	3740.4	125.98
n-Heptane	3788.0	120.41	3791.6	119.74	3803.6	115.96	3798.5	116.25

[a] $\times 10^{-7}$.

TABLE X

EFFECT OF VARIATION OF ENTROPY AND ENTHALPY TERMS ON THE LTPRI VALUES

T_0 = 40.0°C; r = 8.0°C/min. Measured on column 2.

Isothermal (°C)		$-\Delta H/R$ (K)	α/β ($\times 10^{-7}$)	$I_{calc.}$
60.0	*75.0*			
756.62	759.90	3788.2	191.99	750.9
757.52	700.90	3787.0	194.11	751.9
800.00	800.00	4200.9	79.33	800.0
842.88	844.63	4321.9	78.13	840.5
954.92	952.77	5123.3	17.45	952.4
955.60	953.50	5123.9	17.51	953.1

component, 1-butanol. The reason for this close fit is that the entropy and enthalpy terms of each component were calculated based on the adjusted retention times of *n*-alkanes measured at the same inlet pressure condition to be used for the temperature-programmed run, and tabulated Kováts retention indices. This approach ensures the accuracy of the calculated LTPRI's, which is demonstrated further in Table XII for two different columns at different flow-rates for different structural types of substances.

CONCLUSION

For a given stationary phase and a given component at a given temperature, the entropy and enthalpy terms vary according to the film thickness or phase ratio, and

TABLE XI

COMPARISON OF CALCULATED AND MEASURED LTPRI VALUES ON COLUMNS 2 AND 3 AT P_i = 0.450 kg/cm^2

T_0 = 40°C; r = 8.0°C/min. Flow-rate 26 cm/s.

Component	*Column 2*		*Column 3*	
	$I_{calc.}$	ΔI	$I_{calc.}$	ΔI
2-Methyl-2-butene	514.3	0.0	516.3	−0.1
4-Methyl-1-pentene	547.3	−0.1	549.1	−0.1
2,3-Dimethylbutane	557.8	−0.2	557.5	0.1
2-Methyl-1-pentene	583.8	0.0	587.3	0.0
Methylcyclopentane	Co-elute		626.6	−0.1
2,4-Dimethylpentane			623.3	−0.1
Benzene	645.6	−0.1	661.3	0.3
Cyclohexane	655.5	−0.1	Co-elutes with benzene	
2-Methylhexane	661.3	−0.2	Co-elutes with benzene	
1-Heptene	685.7	−0.1	689.5	0.0

TABLE XII

COMPARISON OF CALCULATED AND MEASURED LTPRI VALUES ON COLUMNS 5 AND 6 AT DIFFERENT FLOW-RATES

T_0 = 35.0°C; r = 8.0°C/min.

Component	*Column 5*						*Column 6*					
	Isothermal (°C)		*LTPRI at flow-rate (cm/s)*				*Isothermal (°C)*		*LTPRI at flow-rate (cm/s)*			
	60.0	*80.0*	*38*		*26*		*60.0*	*80.0*	*38*		*26*	
			$I_{calc.}$	ΔI	$I_{calc.}$	ΔI			$I_{calc.}$	ΔI	$I_{calc.}$	ΔI
Benzene	654.37	658.77	643.30	−0.2	645.07	−0.1	669.71	673.97	659.28	0.2	661.00	0.4
Isobutyl acetate	757.79	755.73	754.24	−0.5	754.82	−0.4	774.16	771.94	771.51	−0.1	771.80	−0.1
2-Methoxyethyl acetate	810.99	807.11	811.04	−0.4	810.33	−0.4	838.31	834.21	836.94	0.0	836.48	0.0
Cyclohexanone	860.93	867.57	859.15	−0.4	862.12	−0.3	894.47	900.00	895.34	0.1	897.22	−0.3
2-Ethoxyethyl acetate	882.41	878.15	880.94	−0.2	879.90	−0.3	909.22	904.54	908.02	0.1	906.51	0.0
2-(2-Methoxyethoxy)ethanol	906.07	906.00	905.97	−0.5	906.04	−0.8	934.85	934.77	934.56	−0.1	934.88	−0.2
2-(2-Ethoxyethoxy)ethanol	974.79	974.44	974.47	−0.2	974.62	−0.4						

even the carrier gas flow-rate or inlet pressure. The procedure proposed here utilizes the Kováts retention index, which represents the common properties of a given stationary phase for each component, and the adjusted retention time or capacity ratio of *n*-alkanes, which represents the characteristics of each column. When the reproducibility of the Kováts retention index can be guaranteed, combining these two features (using eqns. 5 and 6) is equivalent to measuring all the components on the particular column. This is why the calculated LTPRI fits the experimental results so well. This procedure can easily be used for interactive database compound identification in linear temperature programmed capillary gas chromatography.

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DETERMINATION OF STOKES RADII OF HYALURONATE OLIGOSACCHARIDES BY SEPHADEX GEL CHROMATOGRAPHY

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SUMMARY

Even-numbered hyaluronate oligosaccharides ranging from di- to hexadecasaccharides were chromatographed on Sephadex G-25, -50 and -75, and their available volumes (K_{av}) were examined. Log K_{av} ratios between any two samples of these oligomers were constant for the three Sephadex types used. The relationships and the use of the log K_{av} of a substance of known size simplified the processes for calculating Stokes radii of hyaluronate oligosaccharides.

INTRODUCTION

Ogston[1] proposed the following equation correlating the available volume (K_{av}) of a spherical molecule with its Stokes radius (R) in a gel:

$$K_{av} = \exp[-\pi L(R + r)^2] \quad (1)$$

where, in the case of Sephadex gel, L is the total concentration of the dextran chain expressed in length per unit volume and r is the radius of a straight dextran chain. Taking the natural logarithm of the above equation gives

$$\log K_{av} = -\pi L(R + r)^2 \quad (2)$$

Using Sephadex gel chromatography, Laurent and Killander[2] demonstrated the usefulness of Ogston's equation in the determination of the spherical equivalent radii (Stokes radius) of proteins and polysaccharides by use of the relationship

$$K_{av} = (V_e - V_0)/(V_t - V_0) \quad (3)$$

where V_e is elution volume, V_0 is void volume and V_t is total bed volume.

We are interested in the conformation of hyaluronate oligosaccharides in solution and have attempted to determine their molecular or Stokes radii in 0.2 *M* sodium chloride solution according to the procedure of Laurent and Killander. For this purpose, even-numbered hyaluronate oligosaccharides were chromatographed on

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some Sephadex gels. In the course of the studies, we noticed that the log K_{av} ratios for any two of these oligomers were independent of the Sephadex type used. These observations accord with eqn. 2. Therefore, by use of the ratios of log K_{av} of hyaluronate oligosaccharides to that of a substance with a known Stokes radius, *e.g.*, cellobiose or bovine serum albumin, the molecular sizes of the oligosaccharides could be determined.

In this paper, we discuss the relationships between log K_{av} values for hyaluronate oligosaccharides on various Sephadex types and a method for determining their Stokes radii.

EXPERIMENTAL

Materials

Even-numbered hyaluronate oligosaccharides, except N-acetylhyalobiuronic acid, were obtained from umbilical cord hyaluronic acid by limited digestion with testicular hyaluronidase (E.C. 3.2.1.35) and characterized as reported previously[3]. N-Acetylhyalobiuronic acid was prepared by treatment of hyaluronic acid with dimethyl sulphoxide containing 10% of 0.2 *M* hydrochloric acid for 16 h at 105°C according to the method of Inoue and Nagasawa[4].

A series of even-numbered hyaluronate oligomers from disaccharide (HA-2) to hexadecasaccharide (HA-16) having an N-acetylhyalobiuronosyl group as the repeating unit and commercial sugars (glucuronolactone, glucose, glucuronic acid, N-acetylglucosamine and cellobiose) were used. Bovine serum albumin (Wako Junyaku Kogyo), chymotrypsinogen and cytochrome *c* (Boehringer), ovalbumin (Sigma) and soybean trypsin inhibitor (Miles Labs.) were also employed.

Gel chromatography

Gel filtration was performed on Sephadex G-15, -25 (fine), -50 (superfine), -75, -100, -150 and -200 (Pharmacia) at room temperature, using 0.2 *M* sodium chloride solution as the eluent. Chromatographic columns (53–56 × 2 cm I.D. and 142–144 × 1.7 cm I.D.) were fitted with a sintered-glass filter disk at the bottom to facilitate the measurement of the total bed volume, V_t. Hyaluronate oligosaccharides and other sugars (about 0.5 mg of each) were applied in most instances as a mixture containing samples separable from each other on columns of various types of Sephadex. The samples were applied in 1 ml of 0.2 *M* sodium chloride solution. Blue Dextran 2000 (Pharmacia) of concentration 3 mg/ml was used to determine the void volume of the column, except for Sephadex G-15, for which hyaluronic acid (0.3 mg) was used. Column eluents were collected in 1.5- or 2-ml fractions, the exact volume of which were determined by measuring the combined volume of ten fractions.

Determination of samples in column eluents

Hyaluronate oligosaccharides, glucuronic acid and glucuronolactone were detected by the carbazole reaction[5]. Neutral sugars, except N-acetylglucosamine, which was measured according to the method of Reissig *et al.*[6], were analysed using the anthrone reaction[7]. Proteins and blue dextran were determined by absorbance measurements at 230 and 620 nm, respectively. The elution volume of sodium chloride on some Sephadex types was examined by adding the salt (150 mg) to the applied

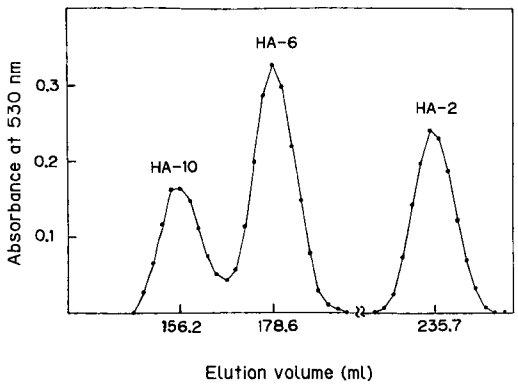

Fig. 1. Chromatography of hyaluronate oligosaccharides on Sephadex G-25. A mixture of HA-2, -6 and -10 was applied on a column (144 × 1.7 cm I.D.) of G-25 and eluted with 0.2 *M* sodium chloride solutes. Fractions of about 2 ml were analysed for uronic acid. The elution volume of Blue Dextran 2000 (V_0) was 141.5 ml.

volume and excess Cl^- concentration above the background of 0.2 *M* was titrated by the method of Fajans.

RESULTS AND DISCUSSION

Gel chromatography of hyaluronate oligosaccharides

The chromatogram of three hyaluronate oligosaccharides (HA-2, -6 and -10) on a long column (144 × 1.7 cm I.D.) of Sephadex G-25 is shown in Fig. 1, in which the elution volume required to elute half of the amount of a substance applied to the column was taken as V_e.

A mixture containing other oligosaccharide samples (HA-2, -4 and -8) was eluted through the same column in a separate experiment. Fig. 2 shows the chromatographic pattern of five hyaluronate oligosaccharides (HA-2, -4, -8, -12 and

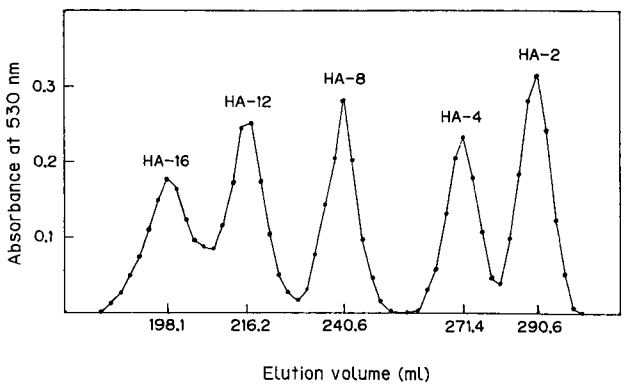

Fig. 2. Chromatography of hyaluronate oligosaccharides on Sephadex G-50. A mixture of HA-2, -4, -8, -12 and -16 was applied on a column (141.7 × 1.7 cm I.D.) of G-50. Assays were performed as in Fig. 1. V_0 was 125.6 ml.

TABLE I

K VALUES FOR HYALURONATE OLIGOSACCHARIDES ON THREE TYPES OF SEPHADEX

The figures in each row were obtained from separate experiments.

Oligosaccharide	*G-25*		*G-50*		*G-75*			
	1[a]	*2*[a]	*1*[a]	*2*[a]	*1*[a]	*2*[a]	*3*[a]	*4*[a]
HA-16			0.37		0.52			
HA-14				0.42		0.56		
HA-12			0.46				0.61	
HA-10	0.079			0.52	0.65			
HA-8		0.11	0.59					0.71
HA-6	0.22			0.66		0.75		
HA-4		0.31	0.74				0.83	
HA-2	0.51	0.49	0.84	0.84	0.89	0.89		0.90

[a] Experiment Nos.

-16) on a column (142 × 1.7 cm I.D.) of Sephadex G-50. The other samples (HA-6, -10 and -14) were co-chromatographed with HA-2 separately. Similar experiments were performed with a column (144 × 1.7 cm I.D., V_0 = 118.1 ml) of Sephadex G-75, in which HA-2 was usually co-chromatographed with other samples as a marker. The results are summarized in Table I in terms of K_{av}, which is hereafter denoted by K.

Relationships among log K for hyaluronate oligosaccharides and bovine serum albumin

Eqn. 2 suggests two important relationships between log K(A) and log K(B) as obtained from the gel filtration of substances A and B on the same gel. For a given Sephadex gel, L in eqn. 2 is constant and

$$\log K(\mathrm{B})/\log K(\mathrm{A}) = \{[R(\mathrm{B}) + r]/[R(\mathrm{A}) + r]\}^2 \quad (4)$$

where R(A) and R(B) are Stokes radii of A and B, respectively. If r is independent of the type of Sephadex gel, log K(B)/log K(A) will be constant for any Sephadex gel which does not exclude either of the two substances.

Next, we compare the K values for a substance A on two Sephadex gels (m and n). If R(A) and r in gel m are identical with those in gel n, the following relationship is obtained from eqn. 2:

$$\log K_n(\mathrm{A})/\log K_m(\mathrm{A}) = L_n/L_m \quad (5)$$

that is, the ratio log K_m(A)/log K_n(A) is determined by the dextran concentrations in gels m and n.

Various log K ratios of hyaluronate oligosaccharides were calculated from the data in Table I and are given in Table II. The log K/log K(HA-2) ratios for a given substance on the three Sephadex gels agree, within the limits of experimental error, but for a series of compounds run on a given gel the ratio increases with increasing chain length. Mean values ± S.D. for log K_{25}/log K_{50} and log K_{75}/log K_{50} were 3.9 ± 0.2 and 0.66 ± 0.02, respectively. The results indicate that eqn. 5 is reasonable.

TABLE II

RELATIONSHIPS AMONG LOG K VALUES FOR HYALURONATE OLIGOSACCHARIDES ON THREE TYPES OF SEPHADEX

Oligosaccharide	*log K/log K(HA-2)*			*log K_{25}/log K_{50}*	*log K_{75}/log K_{50}*
	G-25	*G-50*	*G-75*		
HA-16		5.70	5.61		0.66
HA-14		4.97	4.98		0.67
HA-12		4.45	4.37[a]		0.64
HA-10	3.77	3.75	3.70	3.9	0.66
HA-8	3.09	3.03	3.25	4.2	0.65
HA-6	2.25	2.38	2.47	3.6	0.69
HA-4	1.64	1.73	1.65[a]	3.9	0.62
HA-2	1.00	1.00	1.00	4.0[b]	0.65[b]

[a] The figures were based on the mean (0.893) of the three K(HA-2) values on Sephadex G-75 in Table I.

[b] The ratios (4.0 and 0.65) were calculated by use of the means (0.50 and 0.893, respectively) of K(HA-2) on Sephadex G-25 and -75 in Table I.

Andrews[8] reported on the relationships between elution volume and molecular weight for many proteins on Sephadex G-75 and -100. Each ratio of log K_{75} to log K_{100} on ten proteins was calculated from Andrews' data (Table 3 in his paper[8]), using estimated elution volumes of 74.1 and 96.7 ml for bovine serum albumin on Sephadex G-75 and -100 columns (50 × 2.4 cm I.D.), respectively. The average ratio (0.61 ± 0.03) also supports eqn. 5.

Gel filtration of one or two hyaluronate oligosaccharides together with HA-2 on a short column (53–55 × 2 cm I.D.) of Sephadex G-25 or -50 resulted in similar K values to those on a long column (Table I). Such a short column was convenient for examining the chromatographic behaviour of mixed solutions composed of a few species. By means of this short column, hexadecasaccharide (HA-16) and bovine serum albumin (BSA) were co-chromatographed on Sephadex G-75, -100, -150 and -200. A representative elution profile of the two samples on Sephadex G-150 is shown in Fig. 3. The relationships among log K values for both samples (Table III) also follow eqn. 5.

Determination of molecular radii of hyaluronate oligosaccharides and other sugars

Eqn. 4 can be rearranged to

$$R(\mathrm{B}) = R(\mathrm{A})\,[\log K(\mathrm{B})/\log K(\mathrm{A})]^{1/2} + r\{[\log K(\mathrm{B})/\log K(\mathrm{A})]^{1/2} - 1\} \qquad (6)$$

This equation implies that if R(A), log K(B)/log K(A) and r are known, R(B) can be calculated.

Laurent and Killander[2] assumed that the radius of dextran chains (r) did not depend on the concentration of the polysaccharide; therefore, they used same r value of 0.7 nm for different Sephadex types ranging from G-25 to -200. The validity of their assumption is supported by the result that the log K ratio between two appropriate

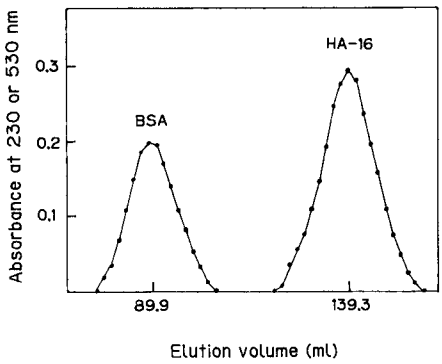

Fig. 3. Chromatography of hexadecasaccharide and bovine serum albumin on Sephadex G-150. A mixture of 1 mg of bovine serum albumin (BSA) and 0.5 mg of hyaluronate hexadecasaccharide (HA-16) was applied on a column (55 × 2 cm I.D.) of G-150. BSA was detected by measuring the absorbance at 230 nm. Assays to detect HA-16 were done as described before. V_0 was 54.9 ml.

samples (Tables II and III) is constant in spite of the use of different gels, as predicted in eqn. 4. Literature values of r, however, spread over a wide range of 0.2–0.7 nm, so the reinvestigation was required. For this purpose, the ratio (3.72) of log K(BSA) to log K(HA-16) shown in Table III and that (1.28) of log K(HA-2) to log K(cellobiose) in Table VI were utilized, because R(BSA) = 3.49 nm[2,9] and R(cellobiose) = 0.51 nm[2,10] are well documented. The following equations were used to calculate r:

$$(3.72)^{1/2} = \frac{R(\text{BSA}) + r}{R(\text{HA-16}) + r} = \frac{3.49 + r}{R(\text{HA-16}) + r} \tag{7}$$

$$(5.66)^{1/2} = \frac{R(\text{HA-16}) + r}{R(\text{HA-2}) + r} \tag{8}$$

$$(1.28)^{1/2} = \frac{R(\text{HA-2}) + r}{R(\text{cellobiose}) + r} = \frac{R(\text{HA-2}) + r}{0.51 + r} \tag{9}$$

TABLE III

LOG K RATIOS FOR HEXADECASACCHARIDE (HA-16) AND BOVINE SERUM ALBUMIN (BSA)

Sephadex	*K*		*log K(HA-16)/ log K_{75}(HA-16)*	*log K(BSA)/ log K_{75}(BSA)*	*log K(BSA)/ log K(HA-16)*
	HA-16	*BSA*			
G-75	0.52	0.087	1.00	1.00	3.73
G-100	0.61	0.17	0.76	0.73	3.58
G-150	0.72	0.30	0.50	0.49	3.67
G-200	0.79	0.40	0.36	0.38	3.89
				Mean ± S.D.	3.72 ± 0.11

TABLE IV

STOKES RADII OF HYALURONATE OLIGOSACCHARIDES

Oligosaccharide	*Stokes radius (nm)*	*Oligosaccharide*	*Stokes radius (nm)*
HA-16	1.70	HA-8	1.21
HA-14	1.59	HA-6	1.03
HA-12	1.48	HA-4	0.83
HA-10	1.35	HA-2	0.60

A value of r to satisfy the above three equations was 0.20 nm, and agreed with that given by Siegel and Monty[11], who postulated dextran as a straight chain and employed this value for all of the Sephadex gels (G-75, -100 and -200) used. With a combination of r = 0.7 nm and R(BSA) = 3.49 nm, the Stokes radius of cellobiose is 0.11 nm from eqn. 7. This value is unacceptable and hence the r value of 0.7 nm could not fit our data. R(HA-2) from eqn. 9 was 0.60 nm on the basis of r = 0.20 nm. By use of these r and R(HA-2) values, and each average ratio [log K/log K(HA-2)] in Table II, molecular radii of the other hyaluronate oligosaccharides were calculated according to eqn. 6. The results are summarized in Table IV. As can be seen, the difference in radius per disaccharide unit tended to decrease slightly with increasing length of the sugar chain. This observation suggests that the segments of oligomer orient more randomly with increase in molecular weight. We noted from Table II a linear relationship between log K/log K(HA-2) and the number of hexose units. The chromatographic data for hyaluronate oligosaccharides reflect their conformations in solution.

Application of the method to some proteins and monosaccharides

Ovalbumin, chymotrypsinogen, trypsin inhibitor and cytochrome c (1 mg of each) were separately chromatographed together with BSA (1 mg) on a column (55 × 2 cm I.D.) of Sephadex G-100. Their Stokes radii, based on R(BSA) = 3.49 and r = 0.20 nm, listed in Table V were in good agreement with literature values.

Although some deviations were found with ovalbumin and cytochrome c, their co-chromatography gave 1.70 nm for the radius of cytochrome c based on R(ovalbumin) = 2.73 nm.

The elution volumes of some monosaccharides involving glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc), which are component sugars of hyaluronic acid, were investigated on Sephadex G-15, -25 and -50. Their K values are summarized in Table VI. The value for HA-2 (0.80) on G-50 was significantly lower

TABLE V

STOKES RADII OF PROTEINS

Protein	*log K/log K(BSA)*	*Stokes radius (nm)*[a]
Ovalbumin	0.705	2.90 (2.73)
Trypsin inhibitor	0.451	2.28 (2.26)
Chymotrypsinogen	0.443	2.26 (2.24)
Cytochrome *c*	0.304	1.83 (1.64)

[a] The Stokes radii given by Laurent and Killander[2] (Table I in their paper) are shown in parentheses.

TABLE VI

STOKES RADII OF MONOSACCHARIDES

Chromatographic data for HA-2, cellobiose (C) and sodium chloride are also shown for comparison. The subscripts a and b indicate different lots of Sephadex G-15. The column sizes for lot a, lot b and G-25 was 55–57 × 2 cm I.D. and that for G-50 was 100 × 2 cm I.D.

Sample	*G-15*				*G-25*			*G-50*		
	K_a	K_b	$log\ K_a/log\ K_a(C)$	$log\ K_b/log\ K_b(C)$	K	$log\ K/log\ K(C)$	$R\ (nm)$	K	$log\ K/log\ K(C)$	$R\ (nm)$
HA-2	0.14	0.22	1.34	1.40	0.51	1.28	0.60	0.80	1.28	0.60
Cellobiose	0.23	0.34	1.00	1.00	0.59	1.00	0.51	0.84	1.00	0.51
GlcUA	0.20	0.28	1.10	1.18	0.60	0.97	0.50	0.85	0.93	0.48
GlcNAc	0.28	0.40	0.87	0.85	0.61	0.94	0.49	0.84	1.00	0.51
Glucose	0.32	0.43	0.78	0.78	0.66	0.79	0.43	0.87	0.80	0.44
Glucuronolactone	0.55	0.65	0.41	0.40	0.77	0.50	0.30	0.91	0.54	0.32
Sodium chloride	0.45	0.56	0.54	0.54	0.76	0.52	0.31	0.91	0.54	0.32

than that (0.84) in Table I. This may be due to the fact that the gel employed in the former experiment was a mixture of two lots. Although the available volumes of sample on lots a and b of G-15 differed from each other in spite of the similar column size, the log K ratio based on cellobiose agreed well; the mean $\pm$ S.D. for log K_b/log K_a of same sugar was 0.74 $\pm$ 0.02. Hence eqn. 5 seems to be satisfied even among different lots of the same Sephadex type.

Faster elution of GlcUA on G-15 compared with cellobiose could be due to repulsion between the negatively charged monosaccharide and the gel matrix. A similar effect was observed in HA-2 having a GlcUA residue. By comparing the available volumes of glucuronolactone and sodium chloride, it was found that the sugar was reversibly adsorbed on the gel surface of G-15. Such a phenomenon was also noted for GlcNAc. The repulsion and adsorption effects were also found in the chromatograms on G-25, in which GlcUA was eluted slightly faster than GlcNAc. Molecular radii of monosaccharides (Table VI) were calculated from eqn. 6 by using log K(cellobiose), R(cellobiose) = 0.51 and r = 0.20 nm. GlcNAc was larger than glucose owing to its bulky substituent and their co-chromatography on G-50 demonstrated a faster elution of GlcNAc. This monosaccharide and cellobiose were found to have similar sizes under the present conditions. Glucuronolactone, with a compact structure, and sodium chloride showed similar mobilities on G-25 and -50. The calculated radius of the salt (0.3 nm) appears to correspond to the sum of their ionic radii (Na^+ and Cl^-). Squire[12] proposed an equation correlating elution volume with molecular weight and calculated the molecular weight of sodium chloride from its chromatographic data on Sephadex G-75. Although the calculated molecular weight showed a negative deviation of 17%, his and our data suggest that gel chromatography on Sephadex can also give information on the molecular weight and molecular size of a small molecule such as sodium chloride.

Sephadex gel chromatography has provided a powerful tool for the determination of the Stokes radius of a substance that does not interact with the gel grains and many investigators[2,12–14] have derived various equations to relate chromatographic data to molecular properties. We have chosen here an equation (eqn. 2 in this paper) given by Ogston[1], because it was useful for explaining our data. In the present method for determining the molecular size of a substance, its co-chromatography with a standard of known size, such as cellobiose or bovine serum albumin, is recommended. The use of the log K ratio was important in eliminating a complicated factor, L, included in eqn. 2 and therefore obtaining a reliable estimate of the radius. This method has the advantage that it needs only, one appropriate standard, but it should be taken into consideration that our calculation is valid when log K ratios of two specified samples in gels of different pore size and of the same material are constant.

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INTERMOLECULAR INTERACTIONS IN THE SORBATE-MODIFIED GRAPHITIZED THERMAL CARBON BLACK SYSTEM

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SUMMARY

Intermolecular interactions between sorbates and graphitized thermal carbon black modified with poly(methyl-β-cyanopropylphenyl)siloxane (OV-225) have been studied. A simple procedure is suggested for the evaluation of the monolayer capacity from the dependence of the specific retention volumes of substances that are incapable of specific intermolecular interactions with modified surfaces on the amount of modifier on the carbon black surface. X-ray spectral microprobe analysis showed that the modifier is distributed uniformly over the whole volume of the granules. Results of a comparative differential method indicated that the modified surface of carbon black remains almost constant at a modifier content of up to 0.8% (w/w) and is the same as that of the untreated material. The modified sorbent possesses specific properties and can be used for the separation of products of the catalytic oxidation of hydrocarbons.

INTRODUCTION

Adsorption modification of the surface of graphitized carbon blacks makes possible the production of various types of sorbents with different selectivities[1–6]. Of special significance is the investigation and utilization of graphitized carbon blacks that contain sufficient amounts of modifying agents to coat the surface as a monomolecular layer. Such sorbents combine the properties of the carbon black surface and those of the modifier[6,7], with their separation abilities being much better than those of capillary columns[7].

This paper reports a further investigation of modified graphitized carbon blacks aimed at producing new adsorbents. Intermolecular interactions between the sorbate and graphitized thermal carbon black (GTCB) modified by poly(methyl-β-cyanopropylphenyl)siloxane (OV-225) were studied.

For the first time the character of the distribution of a modifier over a carbon black granule has been investigated by X-ray spectral microprobe analysis, the principles of which have been published in ref. 8. Also, a comparative differential method, which allows one to eliminate the chemical effect of the surface and determine its area reliably, was applied to determine the surface area of modified GTCB[9,10]. This

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method is based on comparison of nitrogen (krypton) adsorption isotherms of modified and primary standard samples with a macroporous structure and known surface area, determined by an independent method (electron microscopy) in the region of polymolecular adsorption.

The sorbent obtained was applied to the gas chromatographic separation of products of catalytic reactions.

EXPERIMENTAL

Laboratory-made GTCB with a surface area of 8.5 $m^2\ g^{-1}$ and a polar modifier, namely poly(methyl-β-cyanopropylphenyl)siloxane (MW $\approx$ 8000, $\rho = 1.096\ g\ cm^{-3}$)[11], were used.

A weighed carbon black sample was introduced into a solution of OV-225 in chloroform and left there for 48 h for adsorption to proceed. The solvent was then evaporated and the sorbent obtained was sieved, the 0.25–0.5 mm fraction being selected. Adsorbents containing 0.1, 0.2, 0.4, 0.8 and 1.2% (w/w) of modifying agent were prepared.

The capacity of the modifier monolayer was determined chromatographically during the study of the specific retention volumes, $V_{m,1}$, of substances of groups A, B and D (Kiselev's classification[12]) as functions of the amount of modifier on the carbon black surface[4,6,7].

The gas chromatographic and adsorption properties of the modified carbon black samples were investigated with a Khrom-5 chromatograph with a flame ionization detector and stainless-steel column (100 × 0.3 cm I.D.). The specific retention volumes of substances of various classes were measured at different temperatures. As the result, the corresponding differential molar changes in internal energy, $-\overline{\Delta U}_1$, on adsorption, and the contribution of the specific interaction energy, $-\overline{\Delta U}_{1,spec.}$ of the compounds with modified surfaces to the total value of $-\overline{\Delta U}_1$ were calculated.

The distribution of OV-225 over the modified granules was investigated using a JSM-35C scanning microscope supplied with a DDS-35 microanalyser (Si $K\alpha$ line intensity, $\lambda = 7.125$ Å, thallium phthalate as a crystal analyser). The statistical error of the detection did not exceed 1%.

The adsorption properties of the initial and modified carbon blacks were examined using a Digisorb-2600 automatic adsorption installation by measuring the low-temperature adsorption isotherms of nitrogen and krypton.

RESULTS AND DISCUSSION

The dependences of the specific retention volumes of *n*-alkanes (substances of group A), benzene, diethyl ether, acetone (group B) and propanol (group D) on the amount of OV-225 on the surface of GTCB are presented in Fig. 1.

The values $-\overline{\Delta U}_1$ and $-\overline{\Delta U}_{1,spec.}$ for adsorption on the initial GTCB and GTCB modified with 0.4% (w/w) of OV-225 are given in Table I (the choice of this concentration of the modifier is explained below).

The behaviour of the adsorbates studied (Fig. 1) is in good agreement with their energy characteristics (see Table I). For example, on introducing the modifier the

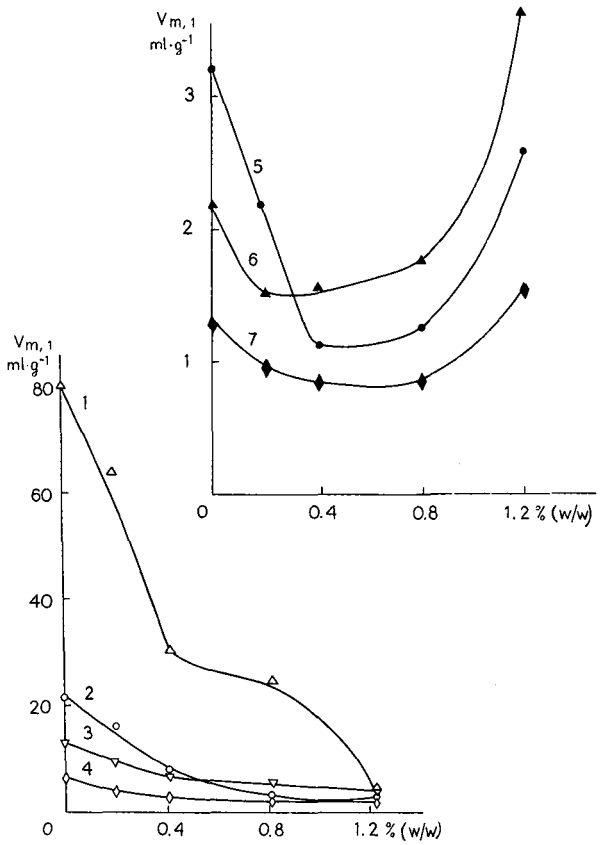

Fig. 1. Specific retention volumes of different classes of substances *vs.* modifier content on GTCB surface at 100°C. 1 = *n*-Heptane; 2 = *n*-hexane; 3 = benzene; 4 = *n*-pentane; 5 = diethyl ether; 6 = *n*-propanol; 7 = acetone.

TABLE I

SPECIFIC RETENTON VOLUMES AND VALUES OF $-\overline{\Delta U_1}$ AND $-\overline{\Delta U_{1,spec.}}$[13] FOR SUBSTANCES OF DIFFERENT CLASSES DURING THEIR INTERACTION WITH THE INITIAL GTCB AND GTCB MODIFIED WITH 0.4% (w/w) OF OV-225

Adsorbate	*Initial GTCB*			*GTCB + 0.4% OV-225*		
	$V_{m,1}$ *(ml/g)*	$-\overline{\Delta U_1}$ *(kJ/mol)*	$-\overline{\Delta U_{1,spec.}}$ *(kJ/mol)*	$V_{m,1}$ *(ml/g)*	$-\overline{\Delta U_1}$ *(kJ/mol)*	$-\overline{\Delta U_{1,spec.}}$ *(kJ/mol)*
n-Pentane	5.18	35	0	2.04	32	0
n-Hexane	20.8	41	0	7.65	39	0
n-Heptane	89.1	48	0	29.4	45	0
Benzene	14.3	38	0	6.14	37	3
Diethyl ether	3.31	32	0	1.12	31	0
n-Propanol	2.20	34	–	1.70	40	16
Acetone	1.33	31	–	0.88	31	9

specific retention volumes of most substances decreased sharply at levels down to 0.4% (w/w). This correlation is most pronounced for substances incapable of specific intermolecular interactions with modified surfaces. However, at a modifier content of up to 0.8% (w/w), the specific retention volumes remain almost constant, decreasing at 1.2% (w/w) of the modifier. Therefore, as in our previous studies[14–16], 0.4% (w/w) is taken as the monolayer capacity.

Using the carbon black surface area of 8.5 $m^2\ g^{-1}$, the monolayer capacity obtained corresponds to specific adsorption of 0.47 mg m^{-2}, which is in good agreement with data for a statistic monolayer of some high-molecular-weight modifiers on the surface of graphitized carbon blacks[4,6,14–18]. Close values of the specific adsorption in monolayers of substances of different chemical compositions are likely to indicate the dominant parallel orientation of fragments of the modifier molecule with respect to the plane of the surface with an average layer thickness of 0.5 nm for OV-225, for example.

Note that a decrease in the specific retention volumes of substances that are incapable of specific intermolecular interaction with the modified surface may depend, in the long run, on a decrease in the surface area of the graphitized carbon black during its adsorption modification[4,7,17]. However, the surface areas of modified GTCB, which were determined by the comparative differential adsorption method[19], remain almost constant up to 0.8% (w/w) of the modifier and equal to that of unmodified GTCB.

The surface areas of untreated and modified GTCBs determined by the comparative method are given in Table II. The values of the C_{BET} constant in the BET equation are also listed.

According to Table II C_{BET}, which characterizes the sorbate–sorbent interactions, decreases significantly on modification of the carbon black surface. A decrease in the specific retention volumes of *n*-alkanes, for example, on the modified GTCB might be explained by the fact that the adsorption field of the surface of graphitized carbon black is due mainly to molecular forces rather than to atomic interactions of surface carbon atoms [4]. Thus, the $-\overline{\Delta U_1}$ values of *n*-alkanes decreased in comparison with the untreated GTCB (see Table I).

However, in contrast to an Apiezon L monolayer supported on GTCB, for which the specific retention volumes of *n*-alkanes decrease by one order of magnitude

TABLE II

SURFACE AREAS (*S*) DETERMINED BY THE COMPARATIVE METHOD AND C_{BET} CALCULATED FROM NITROGEN ADSORPTION ISOTHERMS[19] FOR THE INITIAL AND MODIFIED GTCB

No.	*OV-225 (%, w/w)*	C_{BET}	*S* ($m^2\ g^{-1}$)
1	0	2200	8.3
2	0.1	410	8.4
3	0.2	54	8.6
4	0.4	44	8.6
5	0.8	28	8.0
6	1.2	27	7.6

in comparison with the untreated GTCB[17], the values for an OV-225 monolayer decrease only 2–3-fold.

Such behaviour of *n*-alkanes can be explained, as for salts of sulphonic acids of phthalocyanines of different metals[18], by a much stronger dispersion interaction in the adsorbate–OV-225 monolayer system on the GTCB due to the presence of highly electron-polarized (*N*) atoms in the modifier molecules. As a result, $-\overline{\Delta U_1}$ for *n*-alkanes decreased insignificantly compared with the untreated GTCB (see Table I).

For *n*-hexane, a decrease in the specific retention volume of non-specifically interacting molecules with 1.2% (w/w) OV-225 on GTCB may be attributed to a decrease in the sample surface area (see Fig. 1 and Table II).

For the given modifier (Table I), acetone and *n*-propanol are capable of specific intermolecular interaction even before the surface has been coated by a monolayer. As a result, the observed inflection point on the plot of $V_{m,1}$ *vs.* modifier content of GTCB is shifted towards lower values of the modifier content.

For diethyl ether, this specificity is revealed when the surface coverage exceeds a monolayer. For GTCB with a monolayer of OV-225, $-\overline{\Delta U}_{1,\text{spec.}}$ remains zero (see Table I) and the curve bending is clearest at a modifier content of 0.4% (w/w).

From the results obtained, it follows that the capacity of the modifier monolayer may be evaluated from the $V_{m,1}$ dependences of substances that are incapable of specific intermolecular interaction on the amount of modifier on the GTCB surface. However, the investigation of such dependences for compounds of different classes is especially useful for the purpose of finding the regions where additional specific intermolecular interactions of compounds with modified surfaces take place and, consequently, new sorbent selectivities can be obtained.

It is seen in Table II that on introducing a modifier C_{BET} decreases to 0.4% (w/w). On further additions, C_{BET} remains practically constant. Hence the results of the chromatographic determination of the monolayer capacity are confirmed by the adsorption method.

The distribution of the modifier with CN groups over the support granules was studied by X-ray spectral microprobe analysis using the Si *Kα* line intensity. The beam diameter was approximately 2 μm.

Curves of Si *Kα* intensity variations for modified samples (spectra 1, 2 and 3) along the diameter of the carbon black granule are shown in Fig. 2. As can be seen the modifier is distributed over the whole volume of the granules with some slight

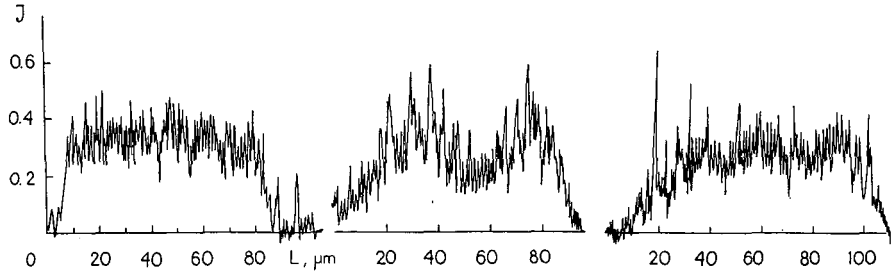

Fig. 2. Intensity of Si *Kα* on scanning three adsorbent granules [0.8% (w/w) OV-225 on graphitized carbon black].

differences in distribution, probably caused by structural differences in the carbon black particles. In Fig. 2 the Si $K\alpha$ line intensity (*ca.* 0.3%) relative to pure silica is indicated without corrections.

Using the ability of the OV-225 monolayer on GTCB to undergo additional specific intermolecular interactions with alcohols, it is possible to separate the products of cyclohexane oxidation. These products include cyclohexane, cyclohexanone and cyclohexanol, which cannot be separated on unmodified GTCB[12]. The results of the separation of products are given in Fig. 3.

Fig. 4 shows a chromatogram of the separation of the catalytic oxidation products of *o*-xylene on a 1 m × 2 mm I.D. column with GTCB modified with 0.8% (w/w) of OV-225. Phthalide, obtained in small amounts, elutes, as on untreated GTCB, before phthalic anhydride and can therefore be determined with sufficient accuracy. In addition, *o*-toluic acid, which is commonly adsorbed on unmodified GTCB, can also be determined with the given adsorbent.

The chromatogram of the catalytic oxidation products of toluene is presented in Fig. 5. Benzoic acid, which is the main product of this process, can also be determined.

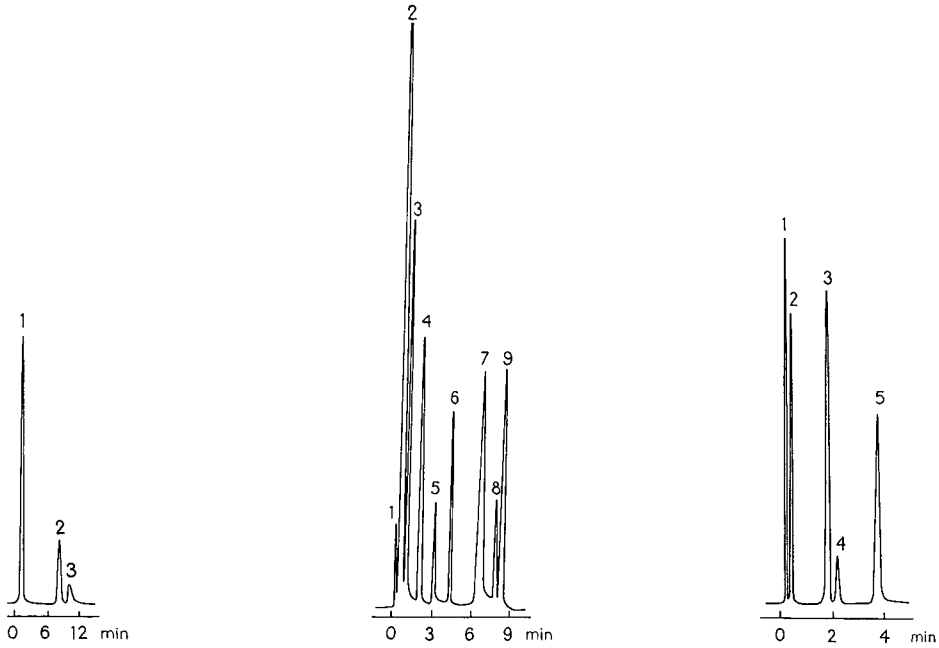

Fig. 3. Chromatogram of cyclohexane catalytic oxidation products obtained in a column with GTCB modified with 0.4% (w/w) of OV-225 at 100°C. 1 = Cyclohexane; 2 = cyclohexanone; 3 = cyclohexanol.

Fig. 4. Chromatogram of products of catalytic oxidation of *o*-xylene to phthalic anhydride obtained in a column of GTCB modified with 0.8% (w/w) of OV-225 with programmed heating of the column from 60 to 180°C at 20°C/min and flame ionization detection. 1 = Air; 2 = acetone; 3 = toluene; 4 = *o*-xylene; 5 = maleic anhydride; 6 = *o*-tolualdehyde; 7 = *o*-toluic acid;8 = phthalide; 9 = phthalic anhydride.

Fig. 5. Chromatogram of products of catalytic oxidation of toluene to benzoic acid obtained in a 1 m × 3 mm I.D. column of GTCB modified with 0.8% (w/w) OV-225 with programmed heating of the column from 65 to 180°C at 32°C/min and flame ionization detection. 1 = Acetone; 2 = toluene; 3 = benzaldehyde; 4 = benzyl alcohol; 5 = benzoic acid.

In general, the adsorbent obtained seems to belong to type III sorbents according to the Kiseler classification[12].

It was shown that for the determination of the monolayer capacity it is sufficient to study the dependence of the specific retention volumes of group A substances on the modifier content on the carbon black surface. For substances in other groups, a study is necessary to establish the regions of additional specific intermolecular interactions and, consequently, to find new possibilities for the separation of different types of substances.

It was found that on adsorption modification of GTCB with these modifiers (up to 0.8%, w/w), the modified surface of carbon black remains almost the same as that of the initial material. This fact is especially important for the determination of the absolute retention volumes of adsorbates, which characterize exclusively the sorbate–sorbent interactions and serve as physico-chemical constants for the identification of components of mixtures. By modifying the surface of GTCB with a monolayer of OV-225, an adsorbent of type III was obtained.

X-ray microprobe analysis was applied for the first time to study the distribution of a modifier over carbon black granules. It was found that the modifier is distributed uniformly over the whole volume of the granules.

The separation ability of the adsorbent obtained differs from that of unmodified GTCB. The modified sorbent has been used for th gas chromatographic analysis of products of the catalytic oxidation of hydrocarbons.

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LIMITING ACTIVITY COEFFICIENTS OF ALIPHATIC ALCOHOLS IN PHTHALATES[a]

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SUMMARY

Limiting activity coefficients of methanol, ethanol, 1-propanol, 1-butanol and 1-pentanol were determined in di-*n*-decyl phthalate (DNDP), diisodecyl phthalate, di-*n*-butyl phthalate and tetrachlorodibutyl phthalate at 50–100°C. In order to ascertain the surface effects at the gas–liquid interface, the limiting values were evaluated at 15, 20 and 30% column loadings for DNDP. The data, corrected for surface effects, show excellent agreement with the experimental data at a 30% column loading. The limiting activity coefficient values of these alcohols were determined at 15 and 30% column loadings for other phthalates. All the alcohols were found to show a positive deviation from Raoult's law. A decrease in the limiting value with increase in the molecular weight of the alcohols was also observed.

INTRODUCTION

Gas chromatography is an excellent method for obtaining equilibrium data at infinite dilution with high accuracy. These data, which indicate the solute–solvent interactions, provide a good source for evaluating UNIFAC group interaction parameters[1,2], which are gaining importance in the design of fluid-phase separation equipment. In fact, the limiting activity coefficient data are very effective both in the description of binary mixtures and in scale-up to multi-component systems.

The limiting activity coefficient characterizes the behaviour of a single solute molecule completely surrounded by solvent molecules. As such, it generally indicates a maximum non-ideality and offers incisive information to the theorist, as the order–disorder effect disappears; it also offers economy of effort to the experimentalist, as the datum has wider applicability than measurement at any other

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concentration. Measurements in this region are difficult by conventional methods and tend to be inherently inaccurate. Conventional methods for studying solution phenomena, which depend on the measurement of vapour pressure, suffer the disadvantage that the experimental error of measurement at low concentrations is usually much greater than at higher concentrations.

Work on solution thermodynamics using gas chromatography commenced in the late 1950s[3–5] and useful data are still being reported. The work of Everett and Stoddard[6] and of Cruickshank *et al.*[7] was found to be more accurate. Kikic and co-workers[8,9] have shown that plots of the logarithm of activity coefficient at infinite dilution (γ^{∞}) *versus* inverse of temperature ($1/T$ K) for aliphatic and aromatic solutes with phthalate ester solvents were not linear, indicating that the excess heat of mixing (Δ_{H_E}) was not constant over the temperature range investigated. Very little information is available in the literature on activity coefficients at infinite dilution of alcohols with respect to phthalates.

In this paper, a gas chromatographic method is described for determining the activity coefficients at infinite dilution of five aliphatic alcohols starting from methanol in four different phthalate stationary phases at different concentrations.

EXPERIMENTAL

Spectroscopic-grade methanol, ethanol, 1-propanol, 1-butanol and 1-pentanol from E. Merck (Darmstadt, F.R.G.) were used.

Chromatographic grade di-*n*-decylphthalate (DNDP), diisodecyl phthalate (DIDP), di-*n*-butyl phthalate (DNBP) and tetrachlorodibutyl phthalate (TCDBP) were obtained from Analabs (North Haven, CT, U.S.A.). These phases were coated on Chromosorb W supplied by Alltech (Arlington Heights, IL, U.S.A.). The coated material was then filled in 243.8 cm × 3 mm I.D. stainless-steel columns. The columns were turned into a spiral shape and fitted in the thermostated oven of the chromatograph maintained at 100°C. Each column was conditioned for 48 h by passing 99.99% pure IOLAR Grade I nitrogen supplied by Indian Oxygen (Bombay, India).

A microprocessor-controlled Hewlett-Packard Model 5840 A gas chromatograph equipped with a thermal conductivity detector was used for determining the activity coefficients at infinite dilution of the solutes with respect to the solvents under study.

Pure hydrogen was used as the carrier gas. The flow-rate was measured by a soap-bubble flow meter to an accuracy of 0.05 ml/min. The carrier gas flow-rates were measured at ambient temperature and corrected to the experimental conditions by means of eqn. 1 (see also p. 16 of ref. 22). After attaining steady conditions in the system, 1 μl of solute was injected and the retention time of the solute, t_r, was noted. Duplicate or triplicate runs were made for each solute. The experiment was then repeated by injecting an inert gas such as methane and its retention time, t_0, was noted.

The specific retention volume, V_g^0, was calculated according to Desty and Swanton[10] by means of the equation

$$V_g^0 = [(JF_m/W)(P_0 - P_{H_2O})/101325](273/T_m)(t_r - t_0) \quad (1)$$

The activity coefficient at infinite dilution (γ_2^∞) of the solute (component 2) was calculated utilizing the relationship between V_g^0 and γ_2^∞:

$$\ln \gamma_2^\infty = \ln(273\ R/P_2^0\ M_1 V_g^0) - (B_{22} - V_2^0)P_2^0/RT_{exp} \quad (2)$$

The second term in eqn. 2 takes into account the non-ideality of the gas phase[11].

The second virial coefficients (B_{22}) of the solutes at required temperatures were calculated as suggested by Hayden and O'Connel[12]. The necessary physical properties of all the solutes, such as vapour pressure (P_2^0) and molar volume (V_2^0), were taken or calculated from ref. 13.

The James–Martin pressure correction factor (J) in eqn. 1 was calculated by using the equation

$$J = \frac{3}{2} \cdot \frac{(P_i/P_0)^2 - 1}{(P_i/P_0)^3 - 1} \quad (3)$$

As gas flow-rates and weights of stationary phases are very sensitive parameters for evaluating the activity coefficients at infinite dilution, they were determined with high accuracy. The flow-rates were checked at the beginning and end of each set of experiments at ambient temperature and were found to be highly consistent. The weights of stationary phases were checked before and after completing the entire set of experiments on a Mettler Model AE-100 balance measuring up to five decimal places. The loss in weight was found to be negligible.

RESULTS AND DISCUSSION

The results for the limiting activity coefficients of all the aliphatic alcohols in DNDP at 15, 20 and 30% solvent loadings are reported in Tables I–III and for 15 and 30 solvent loadings of DIDP, DNBP and TCDBP in Tables IV–IX. In all instances the 30% loading indicates a regular decreasing trend in limiting activity coefficients. Although this trend was observed for the higher boiling solutes 1-pentanol, 1-butanol and 1-propanol even at 15% loading, methanol and ethanol deviated from this trend at 15% loading. The abnormal behaviour of these lower alcohols can be attributed to bulk and surface effects when the solvent loading is below 30%.

The quantitative interpretation of gas chromatographic data is often complicated by the occurrence of solute adsorption at one or more interfaces in the system.

TABLE I

ACTIVITY COEFFICIENTS AT INFINITE DILUTION OF ALCOHOLS IN 13.7% DNDP

Solute	*50°C*	*60°C*	*70°C*	*80°C*	*90°C*	*100°C*
Methanol	1.90	1.86	2.32	255	2.61	3.15
Ethanol	1.76	1.55	1.57	1.82	2.08	2.07
1-Propanol	1.44	1.37	1.32	1.24	1.23	1.27
1-Butanol	1.37	1.32	1.25	1.18	1.07	1.01
1-Pentanol	1.23	1.18	1.11	1.04	0.99	0.89

TABLE II

ACTIVITY COEFFICIENTS AT INFINITE DILUTION OF ALCOHOLS IN 19.7% DNDP

Solute	*50°C*	*60°C*	*70°C*	*80°C*	*90°C*	*100°C*
Methanol	1.74	1.75	2.04	2.42	2.52	2.35
Ethanol	1.55	1.48	1.47	1.59	1.73	1.69
1-Propanol	1.53	1.35	1.26	1.20	1.16	1.24
1-Butanol	1.43	1.26	1.21	1.08	1.05	1.03
1-Pentanol	1.27	1.16	1.09	1.01	0.96	0.94

TABLE III

ACTIVITY COEFFICIENTS AT INFINITE DILUTION OF ALCOHOLS IN 29.8% DNDP

Solute	*50°C*	*60°C*	*70°C*	*80°C*	*90°C*	*100°C*
Methanol	2.31	2.12	2.14	2.09	2.07	1.99
Ethanol	1.91	1.77	1.69	1.61	1.54	1.46
1-Propanol	1.72	1.50	1.36	1.31	1.22	1.18
1-Butanol	1.72	1.48	1.34	1.18	1.11	1.05
1-Pentanol	1.52	1.26	1.16	1.08	1.04	0.97

TABLE IV

ACTIVITY COEFFICIENTS AT INFINITE DILUTION OF ALCOHOLS IN 14.8% DIDP

Solute	*50°C*	*60°C*	*70°C*	*80°C*	*90°C*	*100°C*
Methanol	2.06	2.05	2.46	2.81	2.93	2.79
Ethanol	1.98	1.83	1.83	1.82	2.08	2.05
1-Propanol	1.92	1.75	1.58	1.48	1.34	1.39
1-Butanol	1.80	1.56	1.46	1.31	1.21	1.16
1-Pentanol	1.53	1.38	1.30	1.19	1.11	1.08

TABLE V

ACTIVITY COEFFICIENTS AT INFINITE DILUTION OF ALCOHOLS IN 30.7% DIDP

Solute	*50°C*	*60°C*	*70°C*	*80°C*	*90°C*	*100°C*
Methanol	2.45	2.32	2.35	2.30	2.23	2.16
Ethanol	2.22	2.05	1.89	1.79	1.73	1.64
1-Propanol	1.93	1.73	1.58	1.46	1.36	1.29
1-Butanol	1.86	1.67	1.49	1.36	1.25	1.15
1-Pentanol	1.52	1.37	1.30	1.29	1.20	1.13

TABLE VI

ACTIVITY COEFFICIENTS AT INFINITE DILUTION OF ALCOHOLS IN 15.1% DNBP

Solute	*50°C*	*60°C*	*70°C*	*80°C*	*90°C*	*100°C*
Methanol	2.07	2.01	2.05	2.02	2.01	2.08
Ethanol	2.00	1.76	1.66	1.68	1.65	1.60
1-Propanol	1.86	1.63	1.41	1.31	1.26	1.25
1-Butanol	1.78	1.58	1.44	1.29	1.24	1.13
1-Pentanol	1.64	1.46	1.29	1.20	1.14	1.06

TABLE VII

ACTIVITY COEFFICIENTS AT INFINITE DILUTION OF ALCOHOLS IN 30.6% DNBP

Solute	*50°C*	*60°C*	*70°C*	*80°C*	*90°C*	*100°C*
Methanol	2.53	2.43	2.42	2.38	2.37	2.31
Ethanol	2.19	2.68	2.02	1.98	1.94	1.88
1-Propanol	2.05	1.81	1.67	1.59	1.55	1.53
1-Butanol	2.00	1.89	1.69	1.56	1.43	1.39
1-Pentanol	2.01	1.61	1.48	1.39	1.29	1.27

TABLE VIII

ACTIVITY COEFFICIENTS AT INFINITE DILUTION OF ALCOHOLS IN 13.1% TCDBP

Solute	*50°C*	*60°C*	*70°C*	*80°C*	*90°C*	*100°C*
Methanol	3.49	3.97	4.85	5.17	4.18	3.82
Ethanol	3.22	3.11	3.08	3.38	3.37	3.00
1-Propanol	2.89	2.73	2.43	2.26	2.18	2.17
1-Butanol	2.85	2.61	2.28	2.03	1.84	1.76
1-Pentanol	2.45	2.27	2.02	1.83	1.69	1.72

TABLE IX

ACTIVITY COEFFICIENTS AT INFINITE DILUTION OF ALCOHOLS IN 30.9% TCDBP

Solute	*50°C*	*60°C*	*70°C*	*80°C*	*90°C*	*100°C*
Methanol	3.63	3.89	4.02	4.01	3.99	3.88
Ethanol	2.93	2.72	2.61	2.58	2.48	2.34
1-Propanol	2.72	2.59	2.63	2,44	2.09	2.03
1-Butanol	2.61	2.29	2.11	1.93	1.78	1.67
1-Pentanol	2.35	2.05	1.84	1.71	1.59	1.50

TABLE X

SPECIFIC RETENTION VOLUMES (V_g^0) OF ALCOHOLS IN DNDP

W_1 = weight of DNDP at 13.7% loading (0.3873 g); W_2 = weight of DNDP at 19.7% loading (0.6429 g); W_3 = weight of DNDP at 29.8% loading (0.9362 g); $1/W_1$ = 2582 (kg); $1/W_2$ = 1555 (kg); $1/W_3$ = 1068 (kg).

Temperature	*Methanol*			*Ethanol*			*1-Propanol*			*1-Butanol*			*1-Pentanol*		
(°C)	$1/W_1$	$1/W_2$	$1/W_3$	$1/W_1$	$1/W_2$	$1/W_3$	$1/W_1$	$1/W_2$	$1/W_3$	$1/W_1$	$1/W_2$	$1/W_3$	$1/W_1$	$1/W_2$	$1/W_3$
50	0.050	0.055	0.042	0.103	0.115	0.093	0.296	0.278	0.248	0.744	0.817	0.679	2.219	2.142	1.795
60	0.033	0.036	0.030	0.072	0.075	0.063	0.173	0.189	0.170	0.501	0.502	0.458	1.254	1.274	1.173
70	0.018	0.021	0.020	0.028	0.049	0.043	0.121	0.126	0.117	0.304	0.323	0.291	0.770	0.780	0.733
80	0.012	0.013	0.014	0.029	0.031	0.030	0.083	0.086	0.079	0.212	0.221	0.197	0.491	0.504	0.473
90	0.008	0.008	0.011	0.017	0.019	0.022	0.056	0.059	0.056	0.144	0.147	0.136	0.322	0.332	0.313
100	0.005	0.006	0.008	0.012	0.014	0.016	0.037	0.038	0.040	0.100	0.098	0.094	0.230	0.220	0.213

Solid supports do interact, but they are made inert to most of the solutes. Interactions on the other surfaces cause serious errors. Martin[14] was the first to point out that solute adsorption on the surface of a bulk liquid could markedly affect the retention volumes in gas–liquid partition chromatographic (GLPC) systems. This was further supported by Pecsok *et al.*[15] and directly substantiated by static measurements by Martire *et al.*[16,17], who used a polar stationary phase to assess the Gibb's adsorption effects, which occur only with such solvents. Martire[18] further observed that with certain polar solute–polar solvent systems in which the solute activity coefficients were only around unity, liquid surface excess effects do occur. Pecsok and Gump[19] applied a static method to polar solutes in a non-polar solvent, squalene, and showed that a considerable contribution to the retention volume from Gibbs adsorption effects affected the GLPC system.

Conder *et al.*[20] developed equations for the study of the Gibbs effect. These theoretical equations were further substantiated[21] with the help of experimental data for C_3–C_5 alcohols in squalene.

When there are no surface effects, the net retention volume (V_N) of a solute is generally represented by the equation[22]

$$V_N = K_R V_L \tag{4}$$

where K_R is the solute liquid–gas partition coefficient and V_L is volume of the stationary phase. When gas–liquid interfacial adsorption contributes to retention, eqn. 4 is expanded to

$$V_N = K_R V_L + K_S A_L \tag{5}$$

where, A_L is the liquid-phase surface area, V_N is the net retention volume and K_S is the solute liquid–gas interfacial adsorption partition coefficient. The bulk partition coefficient, K_R, is found by plotting V_N/V_L *versus* $1/V_L$ and extrapolating it to the ordinate. Alternatively, plots can also be made of V_g^0 *vs.* $1/W$ as $V_N/V_L = V_N\rho_L/W = V_g^0\rho_L$ and $1/V_L = \rho_L/W$, where ρ_L is the density of stationary phase in m^3/kg.

In these studies, the specific retention volumes of the aliphatic alcohols were evaluated in DNDP stationary phase at column loadings of 13.7, 19.7 and 29.8% at 50, 60, 70, 80, 90 and 100°C. The results are presented in Table X. V_g^0 was plotted vs. $1/W$ and extrapolated to the ordinate to evaluate the real specific retention volume. Eqns 4 and 5 can be written in terms of V_g^0 as

$$V_g^0\rho_L = K_{R\,obsd.} \tag{6}$$

where $K_{R\,obsd.}$ is the apparent distribution coefficient evaluated from the experimental retention volume data, and

$$V_g^0\rho_L = \frac{A_L K_S \rho_L}{W} + K_R \tag{7}$$

The real K_R values and hence the real γ_2^∞ values can be evaluated from the extrapolated V_g^0 values, which are the intercepts at the ordinate ($1/W = 0$). Typical

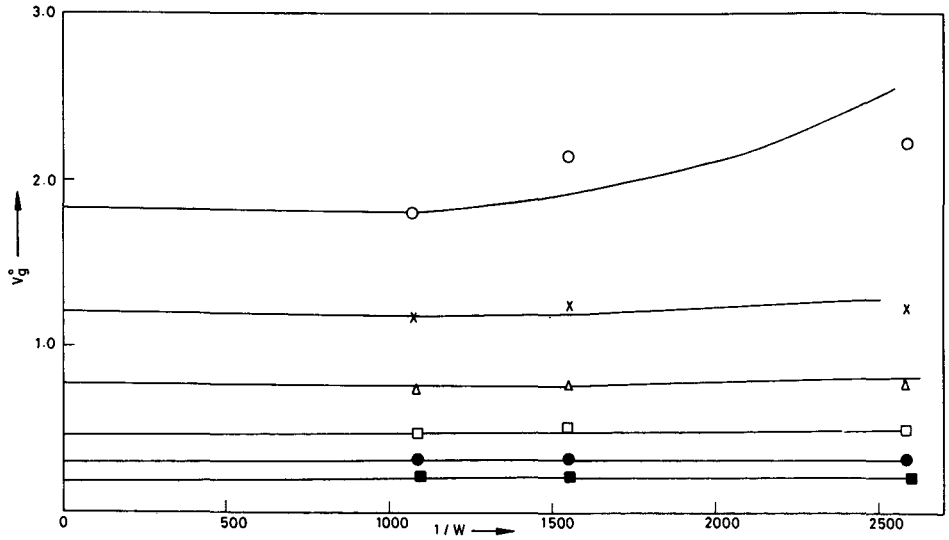

Fig. 1. Specific retention volume of 1-pentanol *vs.* reciprocal of weight of DNDP. ○ = 50°C; × = 60°C; △ = 70°C; □ = 80°C; ● = 90°C; ■ = 100°C.

plots are given in Fig. 1 for 1-pentanol at 50, 60, 70, 89, 90 and 100°C. Similar trends are observed for the other aliphatic alcohols.

The limiting activity coefficients were calculated from these specific retention volumes. The density of DNDP was determined at 25°C to be 1.0083 g/ml.

The real V_g^0 values and γ_2^∞ values are presented in Table XI. They are in excellent agreement with the experimentally observed values presented in Table III. This exercise clearly establishes that the limiting activity coefficients of these aliphatic alcohols are most reliable and accurate. This confirms the work of Martire *et al.*[16], who ascertained that with low-loaded columns surface effects predominate over solubility.

No direct information is available in the literature on the activity coefficients of these alcohols with respect to any phthalate stationary phase. Keulemans[23] evaluated activity coefficients at infinite dilution with respect to DIDP from partition coefficient data[3]. These values were reported at 75, 95, 105, 115 and 135°C. The activity coefficients at infinite dilution as calculated by Keulemans are not in very good agreement with those obtained here, for specific reasons. In their work[3], the specific retention volume was calculated without making a correction for the dead volume as specified in eqn. 1 in this paper. In the reported work, the gas flow-rates were measured at the column temperature without any temperature corrections for ambient conditions. Moreover, the gas-phase fugacity corrections as indicated in eqn. 2 were not applied to the reported data.

The data in Tables I–IX reveal the following effects:

(a) Effect of molecular weight of alcohols: there is a steep fall in the activity coefficients at infinite dilution with increase in the molecular weight of the alcohols. In all instances the activity coefficients at infinite dilution are highest for methanol and lowest for pentanol.

TABLE XI

REAL SPECIFIC RETENTION VOLUMES (V_g^0) AND ACTIVITY COEFFICIENTS AT INFINITE DILUTION OF ALCOHOLS IN DNDP

Solute	*50°C*		*60°C*		*70°C*		*80°C*		*90°C*		*100°C*	
	V_g^0	γ_2^∞	V_g^0	γ_2^∞	V_g^0	γ_2^∞	V_g^0	γ_2^∞	V_g^0	γ_2^∞	V_g^0	γ_2^∞
Methanol	0.041	2.31	0.029	2.16	0.020	2.20	0.014	2.19	0.011	2.06	0.007	2.17
Ethanol	0.091	1.94	0.062	1.80	0.042	1.72	0.030	1.63	0.022	1.56	0.016	1.49
1-Propanol	0.240	1.77	0.170	1.51	0.110	1.44	0.078	1.32	0.052	1.32	0.039	1.21
1-Butanol	0.680	1.71	0.479	1.40	0.299	1.30	0.190	1.26	0.139	1.10	0.091	1.09
1-Pentanol	1.842	1.47	1.220	1.21	0.809	1.06	0.480	1.06	0.308	1.02	0.210	0.98

(b) Effect of temperature: there is a regular decrease in the limiting values for all the alcohols with increasing temperature when 30% solvent loadings are considered. Only methanol shows a peculiar behaviour; the limiting values first increase from 50 to 70°C and finally show a slight decrease. The natural logarithm of the activity coefficients at infinite dilution increases with decreasing temperature, and they converge towards a closer value at higher temperatures. On plotting these values *versus* $1/T$, non-linar behaviour is observed (Figs. 2–5).

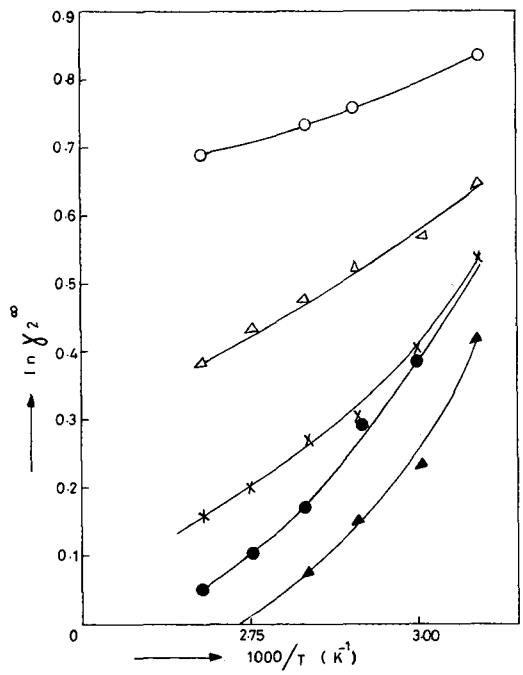

Fig. 2. Activity coefficients at infinite dilution (γ_2^∞) of (○) methanol, (△) ethanol, (×) 1-propanol, (●) 1-butanol and (▲) 1-pentanol in DNDP with a 29.8% column loading.

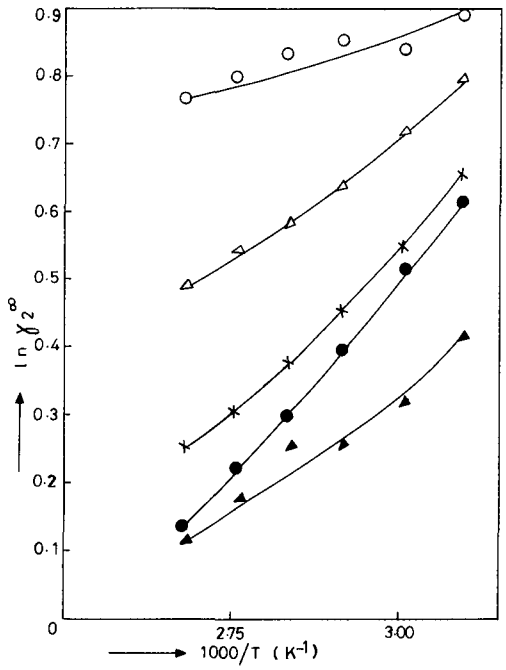

Fig. 3. Activity coefficients at infinite dilution (γ_2^∞) of (○) methanol, (△) ethanol, (×) 1-propanol, (●) 1-butanol and (▲) 1-pentanol in DIDP with a 30.7% column loading.

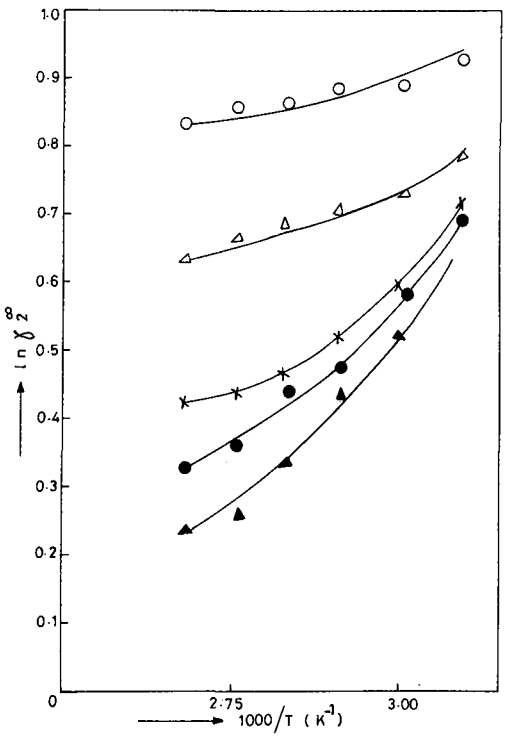

Fig. 4. Activity coefficients at infinite dilution (γ_2^∞) of (○) methanol, (△) ethanol, (×) 1-propanol, (●) 1-butanol and (▲) 1-pentanol in DNBP with a 30.6% column loading.

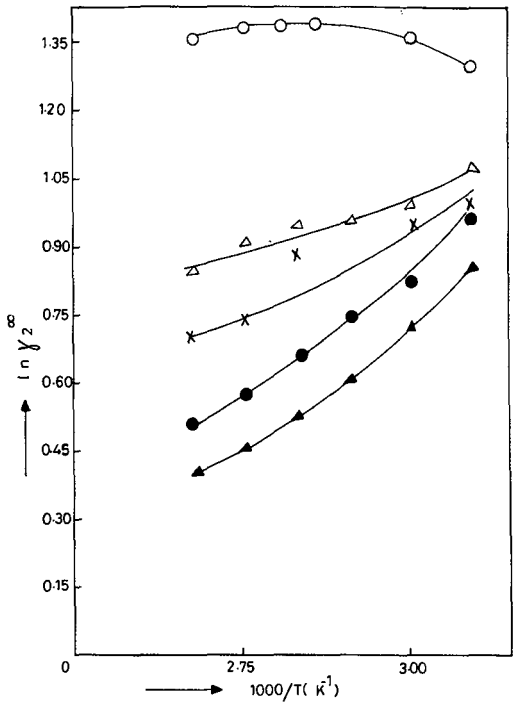

Fig. 5. Activity coefficients at infinite dilution (γ_2^∞) of (○) methanol, (△) ethanol, (×) 1-propanol, (●) 1-butanol and (▲) 1-pentanol in TCDBP with a 30.9% column loading.

(c) Departure from Raoult's law: the interaction parameters (γ_2^∞) of highly pure polar solutes with mild polar solvents do not show a significant deviation from Raoult's law. The maximum deviation occurs with TCDBP. In almost all instances the deviation is positive. The higher boiling alcohols in some instances show a negative deviation at higher temperature.

SYMBOLS

B_{22}	second virial coefficient, m^3/mol
F_m	flow-rate of carrier gas, m^3/s
J	James–Martin pressure correction factor
M_1	molar mass of stationary phase, kg/mol
P_i	pressure at the inlet of column, Pa
P_0	pressure at the outlet of column, Pa
P_{H_2O}	water vapour pressure at T_m, Pa
P_2^0	vapour pressure of solute (component 2), Pa
R	gas constant, 8.314 J/mol · K
T_m	ambient temperature, K
t_r	retention time of solute, s
t_0	retention time of an inert compound, s
T_{exp}	column temperature, K

V_2^0 molar volume of solute (component 2) at T_{exp}, m^3/mol
W mass of stationary phase, kg
γ_2^∞ activity coefficient of solute (component 2) at infinite dilution.

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ANALYSIS OF ORGANIC ISOCYANATES USING CAPILLARY SUPERCRITICAL-FLUID CHROMATOGRAPHY

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SUMMARY

Capillary supercritical-fluid chromatography (CSFC) is shown to provide high-resolution separations of a wide variety of di-, tri- and polyisocyanates, including derived products such as dimer, trimer, urea, biuret, carbodiimide, phenol-blocked, thiophosphoric, carbamate and thiocarbamate isocyanates. CSFC is superior to any other available separation technique for many isocyanates. Derivatization is not necessary with a carbon dioxide mobile phase. Significant changes in selectivity of diisocyanate separations were effected by variations in temperature and use of methyl, biphenyl and octyl methylpolysiloxane stationary phases. Operating temperatures of up to 100°C could be used. With neat carbon dioxide as the mobile phase, the applicable mass range had an upper limit of 500–2000 g mol^{-1}, depending on sample structure and composition.

INTRODUCTION

Polyurethane manufacturing is centred around the reactions of isocyanates with various alcohols. The reactivity of isocyanates and the thermal lability of many of the derived products makes their analysis difficult. The types of isocyanates in use range from the low-molecular-weight to very high-molecular-weight polymers, and also monomeric and polymerized derivatives such as ureas, dimers and trimers, blocked isocyanates and prepolymers of an isocyanate and polyol.

Exposure to low levels of isocyanates can cause serious effects on respiratory and other physiological systems in humans[1]. Therefore, trace analytical techniques have been, and continue to be, extensively studied, with primary emphasis on chromatographic-based methods. Another level of analysis is the screening, or quality control, of bulk product material. Although the synthesis of many monomeric isocyanates can be well controlled, reactions leading to many derived products often yield

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complex mixtures that cannot be adequately characterized by present separation techniques.

The trace analysis of isocyanates in air was recently reviewed[1]. Analysis at trace levels is usually for the determination of the volatile, low-molecular-weight isocyanates that present the greatest exposure hazards. Higher molecular weight isocyanates can be adsorbed on to dust particles, or be present in aerosols, and thereby present health hazards. Allowable exposure limits have recently become based on total isocyanate group concentrations[1], thus including these higher molecular weight isocyanates. Trace chromatographic analysis of isocyanates requires high sensitivity, with sample solution concentrations of the order of 1–100 ppb[2] in high-performance liquid chromatography (HPLC). At these low levels the stability of the isocyanates is a primary concern. To prevent reaction between isocyanates and atmospheric moisture or other compounds that may be present, air samples are usually drawn through a derivatizing/absorbing solution. Derivatization is necessary for HPLC analyses owing to possible reaction with mobile phase components such as water and alcohols.

At the screening level, complications inherent in trace analysis are not present. Both HPLC and thin-layer chromatography are used to analyse derivatized samples, but only with a limited number of the higher molecular weight polymeric isocyanates[1]. Gel permeation chromatography (GPC) provides the only means for analysing many of the higher molecular weight isocyanates. However, moisture, impurities and stabilizers in the GPC mobile phase are potential problems. The low resolution of GPC does not provide much detail about polymeric distributions[3]. Isomeric separations are normally not possible.

The thermodynamic and transport properties of supercritical fluid mobile phases produce higher efficiency separations than are possible in HPLC, and also possess solvating properties not present in gas chromatography (GC). When a low-critical-temperature fluid is used as the mobile phase, such as carbon dioxide (T_c = 31°C), thermally labile compounds may be chromatographed in addition to high-molecular-weight samples. Use of carbon dioxide as the mobile phase should eliminate the need for derivatization of reactive samples such as isocyanates, which is a significant advantage over HPLC. The inherently high resolution of capillary columns when used in supercritical-fluid chromatography (SFC) results in a system that can separate a wide variety of complex mixtures. Most detection systems used in HPLC or GC have been interfaced with SFC. A carbon dioxide mobile phase can be used with a standard GC flame ionization detector, thus allowing the analysis of non-chromophoric samples, a significant advantage over HPLC.

The analysis of toluene diisocyanate (TDI) by capillary column SFC (CSFC) has been reported[4]. The same chromatogram was subsequently published[5] with three peaks indicated to be the dimer, trimer and tetramer of TDI. The monomer and dimer were analysed in this study. No trimer was available for inclusion in this study and, whereas polymerized forms of isocyanates exist, no tetramer structure of TDI has been defined in the literature.

Packed-column SFC (PSFC) is also an extremely powerful separation technique and can solve many analysis problems faster and with higher resolution than is possible with HPLC. However, with isocyanate samples, PSFC may not be a viable alternative, for two reasons. First, the packed columns have a high concentration of surface silanol groups which could react with the isocyanates. Second, an organic

solvent, usually an alcohol, is typically added to the mobile phase to cover these active sites in the column. Such a modifier could also react with the isocyanate samples. However, use of a non-reacting solvent such as a chlorinated hydrocarbon or acetonitrile may be feasible.

The advantageous properties of supercritical fluids were exploited in this study to develop an improved, screening-level separation technique. The effects of temperature and impurities, in both the sample and the mobile phase, were studied with regard to both trace- and screening-level analyses. A wide variety of isocyanate and isocyanate-derived samples were chromatographed. Comparisons with results from GPC were made to estimate the extent of sample elution in CSFC. Samples were tested for thermal stability in GC analysis.

EXPERIMENTAL

Two CSFC instruments were used, one constructed in our laboratories and the other a commercially available instrument. All analyses, except of the IPDI–urea (Fig. 9A), the IPDI–trimer (Fig. 11A) and Desmodur HL (Fig. 11B) were performed with the former instrument.

The former instrument consisted of a high-pressure syringe pump (Model 8500, Varian Instruments, Walnut Creek, CA, U.S.A.) modified for pressure control[6] and a chromatograph oven (Model 4100, Carlo Erba, Milan, Italy) equipped with a flame ionization detector. The injection system consisted of an electrically actuated HPLC sampling valve (Model C14W, Valco, Houston, TX, U.S.A.), with an internal sample loop of 0.2 μl and 1-s injection time, coupled to a CSFC splitter (Scientific Glass Engineering, Ringwood, Australia). The carbon dioxide splitting ratio was approximately 20:1. The valve was fitted with a cooling device to maintain the valve temperature at about 15–20°C. A 2-μm pore screen filter was installed in the sample loading port of the injection valve to prevent contamination or plugging of the chromatographic system by particles from the sample or syringe. The carbon dioxide mobile phase used was 52-grade (99.9992%, Carba Gas, Basle, Switzerland), without dip tube. A neutral alumina trap was installed between the tank and the pump. It consisted of 1.5 m of 1/4 in. I.D. stainless-steel tubing fitted with frits on each end. Valves placed on each end allowed the isolation of the trap from atmospheric moisture following activation at 200°C with a nitrogen purge overnight. The trap was flushed several times with carbon dioxide before filling the pump.

The commercially available CSFC system was a Model 601B from Lee Scientific (Salt Lake City, UT, U.S.A.). It consisted of a syringe pump, oven and flame ionization detector. SFC-grade carbon dioxide (Scott Gases, Plumsteadville, PA, U.S.A.) was used: the cylinder did not have a dip tube.

Columns were 100-μm I.D. deactivated fused silica coated with different stationary phases (Lee Scientific). For all analyses, except the selectivity tests with low-molecular-weight diisocyanates (Figs. 4 and 5), the stationary phase was a 100% methylpolysiloxane (SB-Methyl-100, Lee Scientific) of 0.5-μm film thickness. The stationary phases in Figs. 4 and 5 were biphenyl–methylpolysiloxane (30:70) (SB-Biphenyl-30, Lee Scientific) of 0.25-μm film thickness, and *n*-octyl–methylpolysiloxane (50:50) (SB-Octyl-50, Lee Scientific) of 0.5-μm film thickness, respectively. Short lengths (*ca.* 5 m) were used to decrease the analysis time.

Pressure restriction and column flow in both instruments were regulated with a ceramic frit restrictor (Lee Scientific) (20 cm × 50 μm I.D.) with an original frit length of 2 cm. The frit length was reduced to about 1 cm to provide a linear velocity of about 1 cm s^{-1} with the usual initial programming conditions. Experiments with a 7 μm I.D. capillary restrictor showed no differences in chromatographic profiles. Although the frit restrictor had nearly 10 cm of non-deactivated surface inside the oven, and another 8–10 cm in the detector heated zone, no adsorption was observed.

Data were acquired and processed with Nelson Analytical (Cupertino, CA, U.S.A.) software. The sampling rate for all analyses was 1 or 2 points per second.

Injection solution concentrations were typically 0.02–1.0% in dichloromethane (HPLC grade). A few percent of tetrahydrofuran in dichloromethane was required for complete dissolution of 1,4-phenylene diisocyanate.

The low-molecular-weight diisocyanates were obtained in high purity from various local distributors: isophorone diisocyanate, 2,4-toluene diisocyanate, 1,6-hexane diisocyanate and 1,3-xylylene diisocyanate from Fluka (Buchs, Switzerland), trimethylhexane diisocyanate from Merck (Zurich, Switzerland) and 2,6-toluene diisocyanate and 1,4-phenylene diisocyanate from Aldrich (Steinheim, F.R.G.). Technical grade liquid 4,4′-diphenylmethane diisocyanate was obtained from Merck. All samples of the Desmodur series were obtained from Bayer (Leverkusen, F.R.G.). The Isonate 143L sample was obtained from Upjohn (Kalamazoo, MI, U.S.A.). H2921 (IPDI–urea), T1890/100 (IPDI-trimer) and BF1540 (IPDI–dimer) samples were obtained from Hüls (Marl, F.R.G.). The thiocarbamate adducts are produced by Ciba-Geigy. The idealized or proposed structures are shown in the figures.

Linear density programming was used in all analyses. The programming parameters for analysis of low-molecular-weight diisocyanates (Figs. 2–4) were 5-min initial-density hold time, initial densities (g ml^{-1}) of 0.25 (50°C), 0.18 (75°C) and 0.15 (100°C), 0.01 g ml^{-1} min^{-1} programming rate and a final density of 0.5 g ml^{-1}. All other analyses were made with a density programming rate of 0.03 g ml^{-1} min^{-1} from 0.2 to 0.7 g ml^{-1} after a 5-min initial time (100°C in all instances), except for the Desmodur HL sample (Fig. 11B), which had a final density of 0.75 g ml^{-1}. Final-density hold times were 5–15 min.

GPC analyses were performed with a tetrahydrofuran mobile phase and a polystyrene column. Most of the samples were analysed, except for the low-molecular-weight diisocyanates. Representative gel permeation chromatograms of four complex mixtures are discussed below.

RESULTS AND DISCUSSION

Mobile phase impurity concentrations

Moisture in the chromatographic system, especially in the mobile phase, could lead to reaction with an isocyanate sample. This is clearly detrimental to trace analysis but, depending on the conditions and relative concentrations, may not be significant for screening-level analyses. A comparison was made between impurity levels in a carbon dioxide mobile phase and expected peak concentrations for the two analysis levels, trace and screening (Table I). The peak concentrations can vary by an order of magnitude or more depending on the column dimensions, analysis conditions and retention characteristics.

TABLE I

COMPARISON OF IMPURITY LEVELS IN CARBON DIOXIDE MOBILE PHASE WITH SOLUTE CONCENTRATION

Analysis level	*Solute concentration*[a]		*Carbon dioxide impurities (ppm)*[b]		
	Injection solution	*Peak*[c] *(ppm)*	H_2O	O_2	CO
Screening	1%	100	3	2	5
Trace	10^{-2} ppm	10^{-4}	3	2	5

[a] Assuming a phase ratio of 50 (*e.g.*, 0.5 μm d_f × 100 μm I.D.), capacity factor (k) of 2, injection volume 0.2 μl.

[b] SFC-Grade, Scott Specialty Gases.

[c] Values are not adjusted to account for split injection.

At the screening level the peak concentration is about 100 times that of water and other impurities and would seem to pose few problems. The trace level represents the detection limits of one of the most sensitive HPLC methods for the determination of TDI in air. The method[2] involves both a pre-concentration column and a large injection volume. In this instance the mobile phase impurity concentrations are 1000 times higher than the solute.

Operating temperature

Generally in CSFC it is desirable to operate at as high a temperature as possible in order to decrease the mobile phase viscosity and reduce the elution density, thereby increasing diffusion and obtaining less peak broadening. As many reactions of isocyanates can be driven by heat[7–9], an assessment of the effect of temperature on chromatographic integrity was made. The operating temperature may be a factor in various reactions of isocyanates such as polymerization, dimerization, trimerization, degradation and dissociation. Also, reactions with impurities may be favoured at certain temperatures.

A high-purity sample of 2,4-TDI was chromatographed at 50–100°C with *n*-alkane internal standards. The internal standards were C_{12} and C_{15}, which eluted before and after the TDI, respectively, with baseline resolution at all temperatures. The injection solution concentration was approximately 0.2% in dichloromethane, the concentration of internal standards being slightly higher. Table II lists the ratios of peak areas of the internal standards to 2,4′-TDI in 10°C increments. Clearly, no significant changes occurred in this range. These results contradict a report of dimer and trimer formation above 60–70°C in CSFC[10]. Dimer and trimer formation are reported to be catalysed by heat[7,8,11]; however no details or specific temperatures were reported. The preparation of dimers and trimers is usually performed with the aid of a catalyst[9,12–14]. Underivatized 2,4-TDI elutes at about 132°C in capillary GC with no detrimental effects on quantitation at trace levels[15].

Consideration must be given to two factors in the interpretation of such temperature-dependent results. First, the impurities remaining in isocyanate samples from the manufacturing processes are many and varied. With TDI, they include cyclic and linear ureas, biurets and some higher polyurets and chlorine-containing by-prod-

TABLE II

STABILITY OF 2,4-TDI IN CSFC AT VARIOUS OPERATING TEMPERATURES

Temperature (°C)	*Peak-area ratio*[a]	
	C_{12}/TDI	C_{15}/TDI
50	1.22	1.44
60	1.20	1.43
70	1.21	1.43
80	1.20	1.42
90	1.23	1.42
100	1.23	1.42

[a] *n*-Alkane internal standards.

ucts of the phosgenation steps[16]. If a sample has higher levels of these impurities, then the possibilities for reactions are also greater. Second, the presence of impurities in the mobile phase could also lead to reactions. As described above, the solute concentration relative to mobile phase impurities is important and may pose problems in trace analysis. A different TDI sample, Desmodur T100, apparently contained a few percent of the dimer and a few other impurities, whereas the high-purity TDI sample contained only a nearly undetectable amount of dimer (signal-to-noise ratio 2:1).

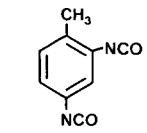
The technical-grade MDI was analysed over a wider temperature range (up to 140°C), although without a detailed internal standard quantification study. No noticeable changes occurred in the profile up to 100°C, except for the expected better peak shape. The temperatures above 100°C may have resulted in decomposition or polymerization.

Selectivity of diisocyanate separations with stationary phase and temperature

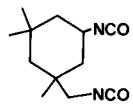
Several common diisocyanates were chromatographed with three stationary phases and at three temperatures. The changes in both peak width and selectivity were significant. The structures are shown in Fig. 1; however TDI, IPDI, and THDI are usually isomeric mixtures. Pure 2,4-TDI was obtained, but formulations are usually 80:20 or 65:35 mixtures with 2,6-TDI, which was also chromatographed. The THDI sample was a mixture of 2,2,4- and 2,4,4-trimethyl isomers. Similarly, the IPDI sample was a mixture of *cis* and *trans* isomers. Nine distinct diisocyanates were present in the sample mixture. Although it is unlikely that all or even most of these diisocyanates would be present in the same sample, several polyisocyanates are based on the combination of two of these, for example TDI and HDI. It is nonetheless advantageous to achieve the separation of as many as possible. No separation of 2,4- and 2,6-TDI was achieved under any conditions. In the following discussion, TDI refers to the coeluting peaks of these two isomers.

Using the non-polar 100% methyl stationary phase, HDI coeluted with the TDI isomers at all temperatures (Fig. 2). These two diisocyanates are used to form a polyisocyanate, Desmodur HL, which was included in this study. The second isomer of THDI and the first isomer of IPDI were slightly separated at 50°C, were well separated at 75°C and had baseline resolution at 100°C.

The biphenyl–methyl (30:70) stationary phase is considered to be a polarizable



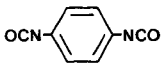
2,4–Toluene Diisocyanate (TDI)



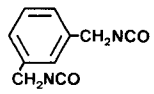
Isophorone Diisocyanate (IPDI)

1,6–Hexane Diisocyanate (HDI)

2,2,4–Trimethylhexane–1,6–Diisocyanate (THDI)



p–Phenylene Diisocyanate (PDI)



m–Xylylene Diisocyanate (XDI)

Fig. 1. Structures of low-molecular-weight diisocyanates.

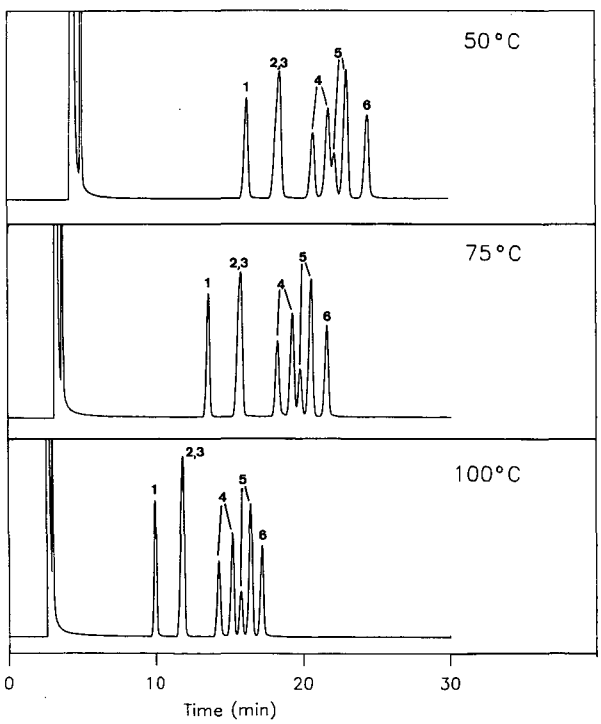


Fig. 2. CSFC traces of a mixture of low-molecular-weight diisocyanates at different temperatures using a 100% methyl stationary phase. Peaks: 1 = PDI; 2 = 2,4- and 2,6-TDI: 3 = HDI; 4 = THDI; 5 = IPDI; 6 = XDI.

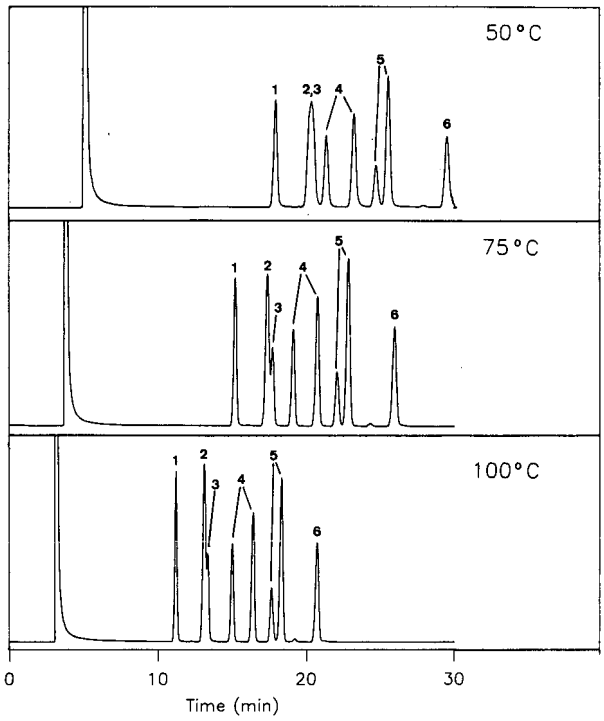


Fig. 3. CSFC traces of a mixture of low-molecular-weight diisocyanates at different temperatures using a biphenyl–methyl (30:70) stationary phase. Peaks as in Fig. 2.

phase [17]. In general, the retention was longest with this stationary phase relative to the other stationary phases (Fig. 3), even though the film thickness was half that of the other stationary phases under study. TDI and HDI again coeluted at 50°C. All other diisocyanates were well separated. At 75°C, HDI was slightly separated from TDI, possibly sufficient for quantitation. At 100°C the resolution of TDI and HDI was poorer than at 75°C.

At 50°C, with an *n*-octyl–methyl (50:50) stationary phase, PDI and HDI were slightly separated, but the resolution may be adequate for quantitation (Fig. 4). TDI and the first isomer of THDI almost coeluted and may yield poor quantitative data. The second isomer of IPDI overlapped slightly with XDI. At 75°C, there was baseline resolution of PDI and HDI, and also between the second THDI isomer and TDI. In contrast, the second IPDI isomer and XDI had poorer resolution, but possibly adequate for quantitation. At 100°C the resolution of all peaks improved, except for the second IPDI isomer and XDI, which coeluted.

The retention behaviour of HDI relative to the other diisocyanates, using the *n*-octyl stationary phase, was interesting and unexpected. As HDI is the most linear of these diisocyanates, it was expected that its retention would increase relative to the other branched alkyl or aromatic diisocyanates. Instead, its retention decreased relative to the other diisocyanates. A possible explanation is that HDI in supercritical

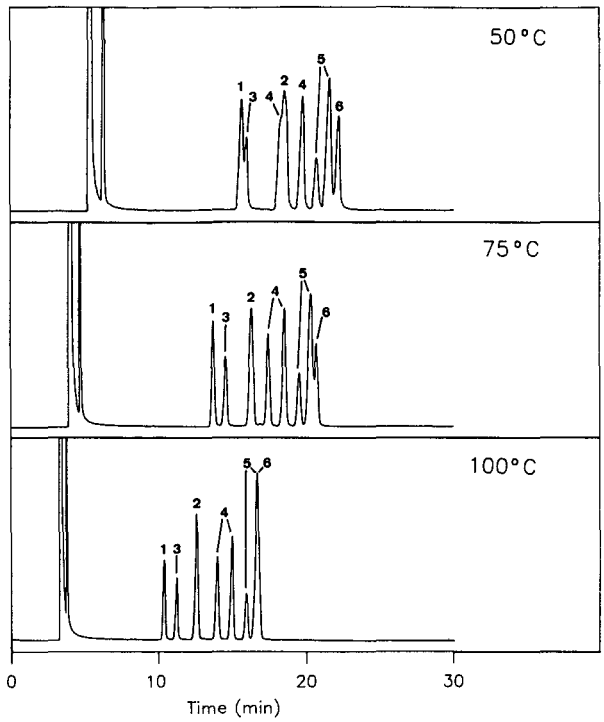


Fig. 4. CSFC traces of a mixture of low-molecular-weight diisocyanates at different temperatures using an *n*-octyl–methyl (50:50) stationary phase. Peaks as in Fig. 2.

solution is not a linear molecule but conforms to another structure which could have a lower relative affinity for the stationary phase.

In general, as the temperature increased the peak widths decreased, as expected. Resolution can also be affected by the mobile phase linear velocity, injection pressure and density, programming rate(s) and column inner diameter and length. Not only have pressure and density programming been demonstrated in CSFC with single-component mobile phases, but also simultaneous density–temperature[18,19] and constant density–temperature[19,20] programming. These features were not utilized in this study, but the former is available in most commercially available CSFC instruments.

MDI-based isocyanates

Recently, 4,4'-diphenylmethane diisocyanate (MDI) and other higher molecular weight isocyanates have been increasingly used as alternatives to the more volatile low-molecular-weight diisocyanates to reduce health hazards. Pure MDI is a solid at room temperature. This fact poses problems in polyurethane manufacture which can be overcome by using MDI modified to remain as a liquid at room temperature. Liquid MDI usually takes one of two forms. The unpurified product containing a series of higher oligomers, as shown in Fig. 5, can be used (polymeric MDI). Alternatively, MDI can be converted into a carbodiimide in the presence of special cata-

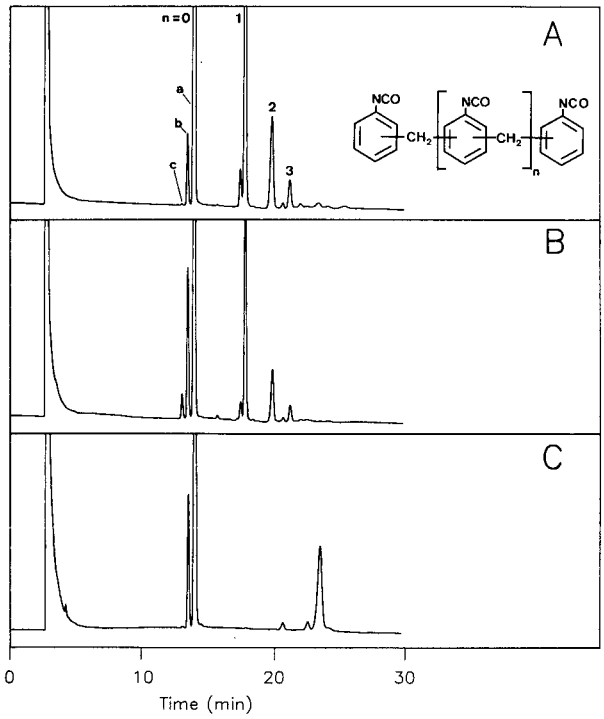


Fig. 5. CSFC traces of liquid MDI products. (A) Technical-grade MDI; (B) Desmodur VL; (C) Isonate 143L. Isomers: (a) 4,4′-MDI; (b) 2,4′-MDI; (c) 2,2′-MDI.

lysts[12,16,21] and added to pure MDI to produce a liquid. Both types of liquid MDI were analysed in this study.

Three samples of liquid MDI were included in this study. The technical-grade MDI and Desmodur VL samples were polymeric MDI, whereas Isonate 143L was a carbodiimide-modified MDI. These three samples were chromatographed at 100°C (Fig. 5). Differences in isomeric and oligomeric compositions were readily apparent. The first grouping of peaks corresponds to MDI, in the polymeric structure $n = 0$. It is known that the major product is 4,4′-MDI, and the 2,4′- and 2,2′-MDI isomers are present in smaller amounts[7,16]. The chromatogram of Desmodur VL (Fig. 5B) provides the best example of the presence of these isomers. Assuming an elution order of 2,2′-, 2,4′-, 4,4′-MDI, then the peak areas correspond to the approximate relative concentrations of the isomers. Oligomers of the polymeric MDI series could be detected up to $n = 4$ or 5, corresponding to molecular weights of 774 and 905 g mol^{-1}. Other peaks were also present and may be alkylated derivatives of the oligomers. Also, one of the peaks may be the dimer of 4,4′-MDI, which can form on standing at room temperature[7,9,16]. Separate homologous series apparently begin with the 2,4′- and 4,4′-MDI oligomers. However, the retention times of the two series converge at $n = 2$, although at 50°C the $n = 2$ peaks could be slightly separated. Such detailed analysis of isomeric distributions is not possible using GPC (Fig. 6).

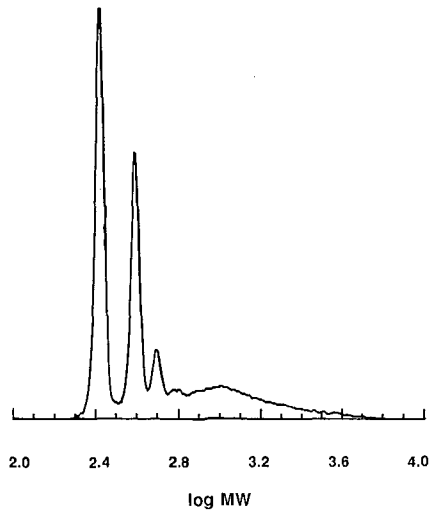
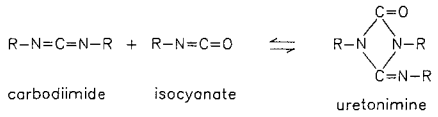


Fig. 6. GPC trace of a liquid MDI product.

As described above, MDI can be converted into a liquid by the addition of the corresponding carbodiimide. At room temperature the carbodiimide is in equilibrium with the corresponding uretonimine formed with free MDI:



No specific information was found in the literature regarding the temperature dependence of the carbodiimide–uretonimine equilibrium, other than the general statement that at higher temperatures the uretonimine dissociates into isocyanate and carbodiimide[16,22,23]. The Isonate 143L sample was initially chromatographed at 100°C (Fig. 5C), but the question remained of whether the peak eluting at higher density (23 min) was the carbodiimide or the uretonimine. The first two peaks corresponded to those of the other MDI samples and are therefore the 2,4′- and 4,4′-MDI isomers. The peak at 23 min was tentatively identified as the carbodiimide. The sample was chromatographed from 35 to 175°C to determine if the suspected carbodiimide peak was in fact a uretonimine. If the carbodiimide were in equilibrium with the uretonimine in the mobile phase, then an increase in temperature should shift the equilibrium towards the carbodiimide, thereby changing the peak-area ratios. The ratio of the area of the suspected carbodiimide peak to that of the 2,4′-MDI peak remained constant up to 100°C, indicating no changes in the suspected carbodiimide. Above 100°C the chromatographic profile changed and additional peaks appeared, and above 150°C the carbodiimide was no longer present. These results indicated that the uretonimine was not present in the Isonate 143L sample when eluted from the column. However, it is possible that the uretonimine was not soluble in carbon di-

oxide and therefore did not elute but only decomposed at the higher temperatures. An answer to this question might be obtained by using infrared spectroscopy with the sample in supercritical solution. However, carbon dioxide absorbs in the same spectral region as the isocyanate group[24], which would interfere with such a study.

Triisocyanates

Three triisocyanate samples were chromatographed at 100°C (Fig. 7), Desmodur R, Desmodur RF and Desmodur L. The peak on the tail of the solvent in Fig. 6A and B was probably phenyl isocyanate. When a concentrated solution of Desmodur R (*ca.* 20%) was injected, the two major peaks could still be distinguished, and were naturally overloaded. About 15 other peaks were also visible, which were probably various by-products. If the analysis cannot be performed at trace levels owing to problems with impurities or insufficient sensitivity, then a more concentrated injection solution will yield information about the minor sample components. However, injection of such concentrated samples could plug either the split or column restrictor.

Reaction of trimethylolpropane (TMP) with 2,4-TDI produces a carbamate triisocyanate which has lower volatility than diisocyanates. At 100°C, the analysis of such a sample, Desmodur L, resulted in the separation of two major and at least six

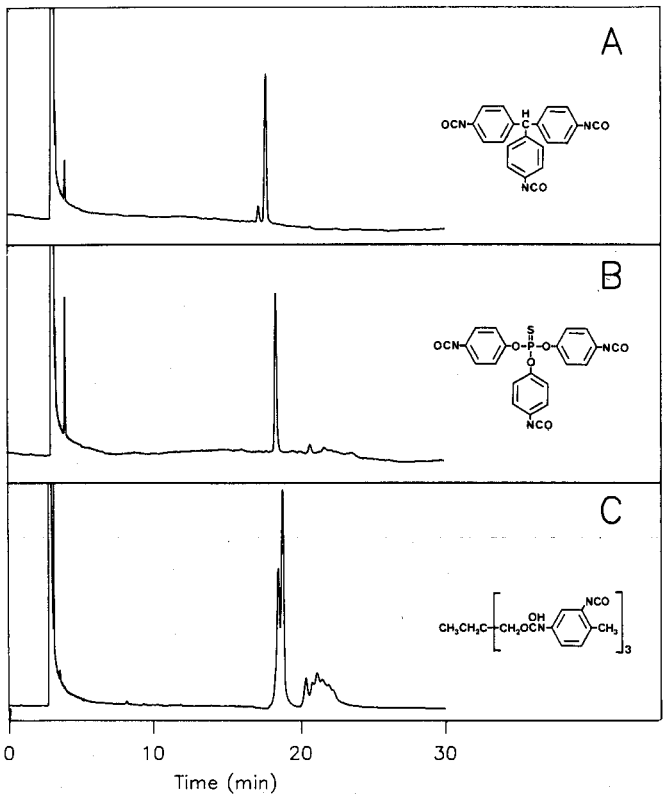


Fig. 7. CSFC traces of triisocyanate products. (A) Desmodur R: (B) Desmodur RF; (C) Desmodur L.

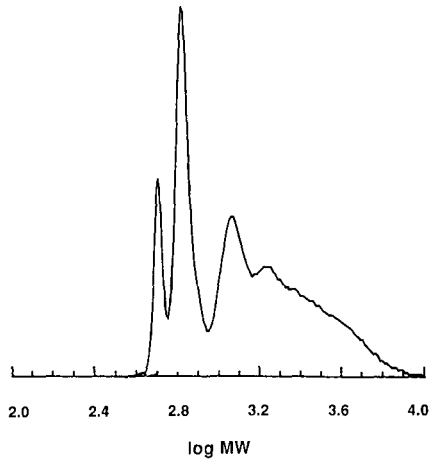


Fig. 8. GPC trace of the triisocyanate Desmodur L.

minor peaks (Fig. 7C). GPC analysis (Fig. 8) of Desmodur L indicated the presence of a component probably composed of TMP and two molecules TDI (referred to as the di-TDI adduct in the following discussion), the proposed product, a component with a molecular weight of about 1200 g mol^{-1}, and of some even higher molecular weight components. Comparison with the SFC trace indicated that the two closely eluting peaks at about 19 min are the di-TDI adduct and the proposed product, and the group of peaks at 20–23 min are the proposed product with additional substitutions, probably TDI. If the group of peaks at 20–23 min were the product then it would not be possible to explain the presence of six peaks in the group based on isomeric distribution, even considering the possible presence of both 2,4- and 2,6-TDI.

Urea and biuret isocyanates

Isocyanates can be converted into ureas, biurets and higher polyurets[16]. Most such derivatives are not amenable to analysis by GC owing to thermal lability. The chromatogram of an IPDI–urea adduct (H-2921) is shown in Fig. 9A. The sample contained about 40% IPDI[25]. The small peaks eluting at about 8 min were also present in the uncatalysed thiocarbamate reaction product discussed later (Fig. 14A). At least four peaks were separated at higher density in Fig. 9A. The major peak was assumed to be the urea. The relative peak area of the urea to IPDI was the same at 50 and 100°C, indicating no losses from the potential reaction of urea with free isocyanate. The IPDI–urea has a molecular weight of 400 g mol^{-1}. The small peaks eluting after the urea may be biuret adducts (*ca.* 642 g mol^{-1}), indicated by comparison with GPC results.

The substituted-biuret sample Desmodur N, formed from HDI, was chromatographed at 100°C (Fig. 9B). Tentative peak identifications were made by comparison with GPC results. A small amount of HDI was still present (peak 1), together with a significant amount of biuret. The proposed substituted-biuret structure, probably peak 4, has a molecular weight of 478 g mol^{-1}. The later eluting peaks (19.5–22

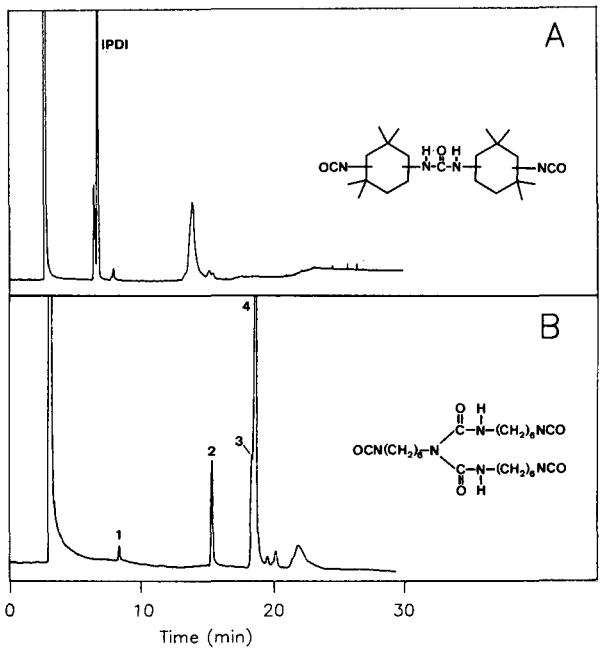


Fig. 9. CSFC traces of isocyanate–urea-derived products. (A) IPDI–urea, H-2921; (B) substituted biuret, Desmodur N. Peak numbers in (B): (1) HDI; (2) biuret; (3) unidentified; (4) probable substituted biuret.

min), presumably higher polyurets, would have molecular weights of 646, 814 and 982 g mol^{-1}, corresponding to the addition of one, two and three HDI molecules, respectively, to the substituted-biuret structure. The results from GPC analysis indicated the presence of the 982 g mol^{-1} of polyuret, which is probably the peak at 22 min. Peak 3 could not be identified. The sample was also chromatographed at 50°C. In that case, resolution was higher between the major peak (4) and the peak eluting immediately before it (3), but was poorer between the major peak and later eluting peaks.

Uretedinedione isocyanates (dimers)

The common name for a uretedinedione is a dimer. The uretedinedione structure is noted to decompose above 130–150°C[11]. The dimer of 2,4-TDI, Desmodur TT, was chromatographed at 100°C (Fig. 10A). A small impurity was present that eluted after the major peak. The sample was also chromatographed at 150 and 175°C. There was slight decomposition at 150°C and almost complete decomposition at 175°C.

Another sample, BF1540, was described as the dimer of IPDI with dimerization occurring on the primary isocyanate. Aliphatic isocyanates do not generally form dimers[9,16], although trimers can be formed. The sample was chromatographed at 50–100°C in case the aliphatic uretedinedione structure was less thermally stable than the corresponding aromatic dimer, but the relative peak areas remained constant. At 100°C (Fig. 10B), at least ten major peaks were present. There appeared to be two

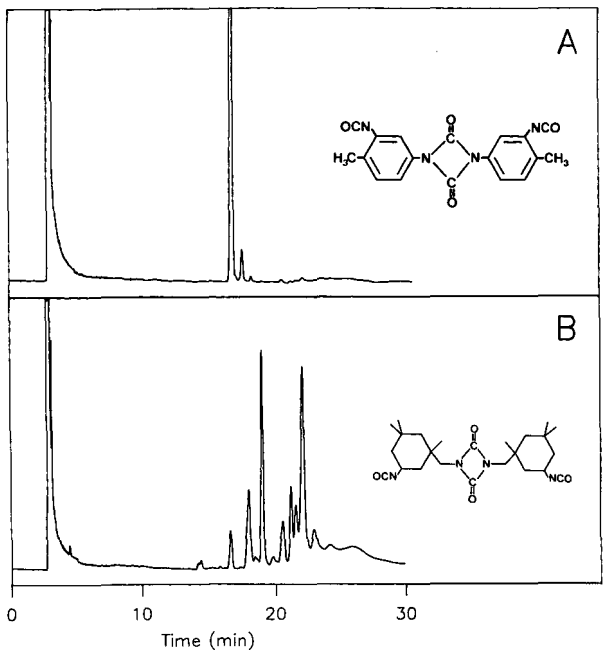


Fig. 10. CSFC traces of uretedinedione products of (A) 2,4-TDI, Desmodur TT; (B) IPDI, BF1540.

series of peaks, the first containing three major peaks of increasing concentration and the second 3–5 major peaks. There was also one very broad peak eluting at the higher density. The first series of peaks eluted with good profiles at the lower temperatures, but the latter peaks had very poor shape except when the temperature was 80°C or higher. The best results were obtained at 100°C. Although infrared spectroscopic analysis of BF1540 indicated the presence of the dimer group, there was very little free isocyanate, which indicated that the sample was highly polymerized. Comparison with GPC results indicated that components of up to 1500–2500 g mol^{-1} were eluted in CSFC. No definite peak identifications could be made.

Isocyanurate isocyanates (trimers)

The common name for an isocyanurate is a trimer; both aromatic and aliphatic isocyanates can form trimers[9,16]. The isocyanurate ring is the most thermally stable of the isocyanate-derived products included in this study. Polymer additives containing the isocyanurate ring have been chromatographed by CSFC at 140°C[26] and by high-temperature GC[27].

The trimer of IPDI, T1890/100, was chromatographed at 100°C (Fig. 11A). A series of five peak groups were present, which apparently constitute an homologous series. The first group contained three large and one small peak and the second group was barely separated into two peaks. These secondary series of peaks may arise from reactions of the primary and secondary isocyanate groups of IPDI, or from the *cis* and *trans* isomers. Comparison with GPC results (Fig. 12) indicated the largest group of peaks (15 min) was the trimer, and that the later eluting peaks corresponded to up

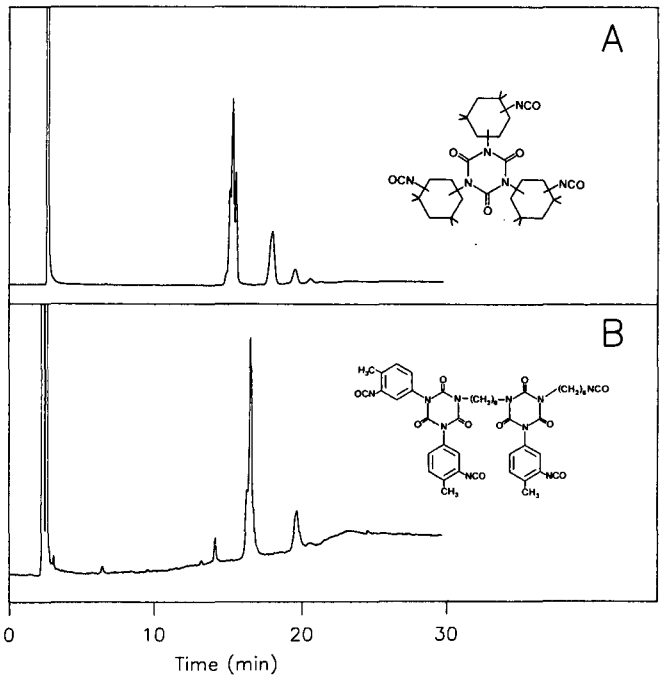


Fig. 11. CSFC traces of isocyanurate products. (A) IPDI–trimer, T1890/100; (B) Desmodur HL.

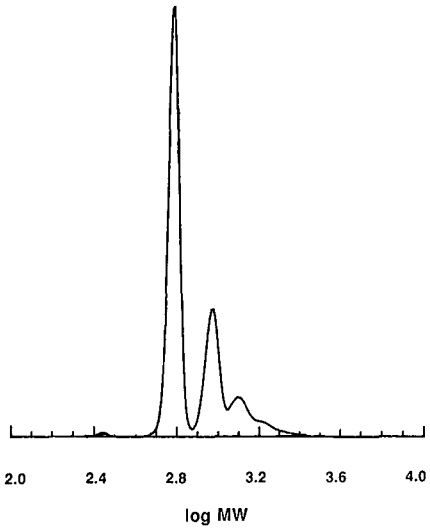


Fig. 12. GPC trace of the IPDI–trimer T1890/100.

to four additional IPDI molecules attached to the trimer. The highest molecular weight would be 1554 g mol^{-1}

One sample which is not a trimer but a high-molecular-weight polyisocyanate possessing two isocyanurate rings is Desmodur HL. The molecular weight of the proposed structure is 858 g mol^{-1} (Fig. 11B). CSFC analysis resulted in the elution of several peaks (Fig. 11B). A small amount of TDI and/or HDI was present (6 min). Three peaks at about 17 min were barely separated; these were probably combinations of TDI and HDI in a single isocyanurate ring (indicated by comparison with GPC). The peak at 20 min could be identified by comparison with results from GPC analysis as having a molecular weight close to the proposed structure. The molecular weight data from GPC analysis were not sufficiently precise to determine the exact structure of the eluted component.

Phenol-blocked isocyanate

A blocked isocyanate is one that has been reacted with any of a number of possible reagents, and that will decompose to the original products (isocyanate and blocking agent) on heat treatment. Phenol-blocked isocyanates will decompose to isocyanate and phenol at about 120°C[28], and are the most widely used. The trifunctional isocyanate Desmodur L (Fig. 7C) can be obtained as the phenol-blocked derivative, Desmodur AP, which was chromatographed at 100°C (Fig. 13). A single major peak was eluted, with a minor peak at higher density. Phenol was also present, eluting just after the solvent. The phenol was also detected when chromatographed at 50°C. Above 120°C, the major peak disappeared. No identifications could be made by comparison with results from GPC owing to dissimilar profiles.

Thiocarbamate adducts

Two different thiocarbamate products formed from the combination of IPDI and a mercaptosilane were chromatographed, one where a catalyst was used and one

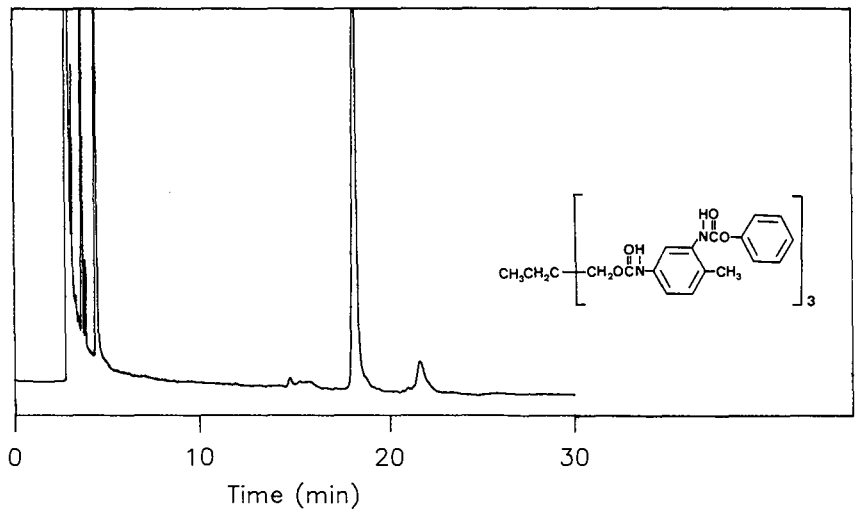


Fig. 13. CSFC traces of the phenol-blocked isocyanate product Desmodur AP.

without a catalyst (Fig. 14). Thiol–isocyanate reaction products are generally more thermally stable than the corresponding urethane adducts. In both samples the remaining IPDI was apparent, and in the uncatalysed product (Fig. 14A) a small amount of the thiol was still present. In the catalysed product (Fig. 14B) there were two major product peaks, but each appeared to be two peaks. This was indicated by the presence of a shoulder on the second product peak when chromatographed at 50°C. In the uncatalysed product (Fig. 14A) several other peaks were present in addition to the major product peaks. An apparently homologous series up to $n = 5$ or 6, possibly additional IPDI substitutions on the thiocarbamate, were eluted in this chromatogram. At higher sample concentration, oligomers up to $n = 8$ could be detected, of molecular weight nearly 2000 g mol^{-1}, determined by comparison with GPC results (Fig. 15).

The additional early eluting peaks, between 11 and 14 min, with the uncatalysed sample (Fig. 14A) could not be immediately identified. They were tentatively identified as the dimer or trimer of IPDI, or possibly ureas. None of these peaks corresponded to any of the peaks in the IPDI dimer sample BF1540 (Fig. 10B). The group of peaks at about 11 min was also present in a small amount in the IPDI–urea sample (Fig. 9A), but the urea was the later eluting peak in Fig. 9A. The retention patterns and relative peak areas between these unidentified peaks and the IPDI–trimer sample (Fig. 11A) were similar, but the elution densities were substantially different. No definitive peak identities could be made without confirmation by mass spectrometry. However, the most reasonable explanation is that the unidentified peaks in the uncatalysed sample were IPDI dimer, that the IPDI–urea and trimer structures (Fig. 9A and 11A) were as assumed and, hence, that the BF1540 sample (Fig. 10B) did not

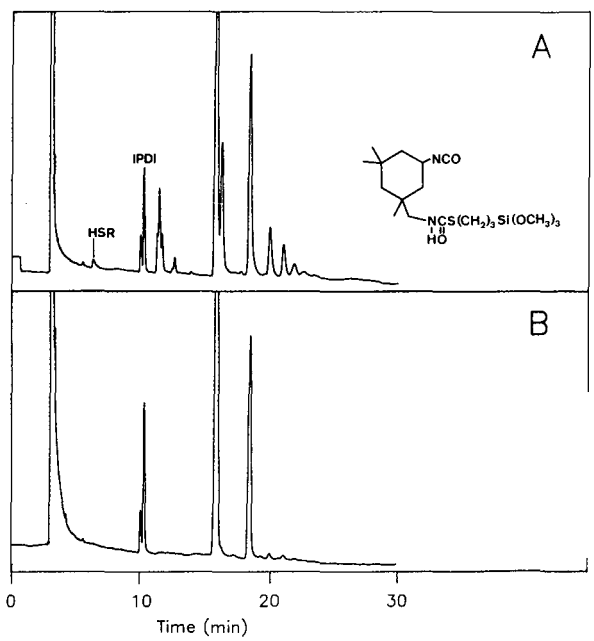


Fig. 14. CSFC traces of thiocarbamate adducts. (A) Uncatalysed and (B) catalysed reaction products.

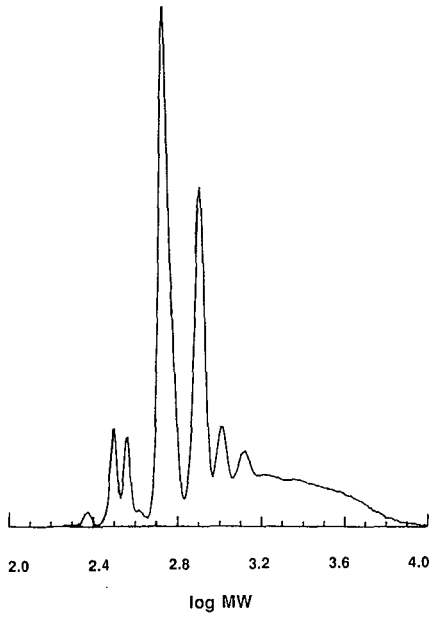


Fig. 15. GPC trace of thiocarbamate adduct (uncatalysed reaction product).

contain the dimer of IPDI. In addition to the IR data noted above, GPC analysis of the BF1540 sample also indicated that no dimer was present. This explanation would also correspond better to the general trend of higher elution density with higher molecular weight.

Gas chromatographic analysis

All samples were tested for compatibility with gas chromatography (GC) using cold, on-column injection and a non-polar stationary phase. Some of the low-molecular-weight diisocyanates have been analysed underivatized at trace levels[15,29], and all diisocyanates in this study eluted without decomposition. MDI has been demonstrated to be amenable to GC[30,31]. Whereas MDI eluted well in GC, higher oligomers did not elute in this study, although some injections produced peaks for the oligomer of $n = 1$. The Isonate 143L sample produced peaks for the MDI oligomer, but the major peak tailed severely and no other peaks were present, which would have corresponded to the carbodiimide. The triisocyanate Desmodur R could be eluted with good peak shape. All other samples decomposed during GC analysis.

GPC analysis

The GPC profiles of polymeric MDI products, thiocarbamate adducts, IPDI–trimer and IPDI–urea corresponded well with the CSFC results. This indicated that all or nearly all of each sample eluted with CSFC. For the samples Desmodur N, L and AP, it appeared that only 50–70% of the samples eluted with CSFC. For two samples, the IPDI–dimer BF1540 and Desmodur HL, it appeared that only about one third of the samples eluted with CSFC. The non-eluting fractions of these samples were represented in GPC by broad distributions of up to 10 000 g mol^{-1} or more.

Chromatographic degradation from highly polymerized isocyanates

With polymeric isocyanates or isocyanate–polyol prepolymers there may exist homologues of extremely high molecular weight, of 10 000 g mol^{-1} or more. Many lower molecular weight isocyanates will polymerize on standing. In CSFC it is always desirable to elute all of an injected sample. However, it is possible that only isocyanates up to certain molecular weights will elute with a given mobile phase. In this instance there will be a build-up of sample on the column, necessitating measures such as the use of precolumns or cutting off part of the column occasionally during extended, routine analyses. An undesirable situation would develop when other types of samples are injected on to the same column, such as alcohols. This could easily lead to polyurethane formation on the column, which would be detrimental to quantitation, consistency in selectivity and extended column lifetimes. No chromatographic degradation was observed in this study. A supercritical-fluid extraction–injection system which would transfer only those components of a sample that are soluble in the mobile phase to the column would be extremely valuable, both for these samples and for many other high-molecular-weight samples analysed by either packed or capillary column SFC. Alternatively, a different mobile phase, either mixed or neat, may elute the higher-molecular-weight isocyanates that cannot be eluted with carbon dioxide.

CONCLUSIONS

The results demonstrate that CSFC provides more information regarding sample composition than any other available chromatographic technique, especially for polymeric isocyanates, or where mixtures of isomeric and/or by-products are present. Further improvements in resolution can be obtained using longer (10–20 m) and smaller I.D. (50 μm) columns, and varying the programme types and parameters. The higher degree of polarizability of the isocyanates produced strong interactions with the biphenyl stationary phase. With several samples not all of the sample components eluted with a carbon dioxide mobile phase. With a neat carbon dioxide mobile phase the applicable mass range had an upper limit of 500–2000 g mol^{-1}, depending on sample structure and composition. The most reasonable estimates of peak identities were made on the basis of known or proposed structures, elution profiles and comparison with results from GPC. However, confirmation of peak identities will require analysis by SFC–mass spectrometry.

Operating temperatures of up to 100°C could be used with a carbon dioxide mobile phase when analyses were performed at a screening level. However, the possibility of temperature-catalysed reactions of isocyanates with themselves or impurities in either the sample or mobile phase cannot be excluded for all possible samples. The purity of the mobile phase will require additional study if trace analysis is to be performed with CSFC.

The successful separations demonstrated in this study indicate the range of isocyanate samples that are soluble in neat carbon dioxide. Although the insolubility of the highly polymerized sample fractions is detrimental to chromatographic analysis, these differences in solubility could be exploited in the development of fractionation and extraction processes. Samples of isocyanates, isolated from polymerized fractions, could yield a higher degree of control over isocyanate reactions.

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EVALUATION OF SUPERCRITICAL SULFUR HEXAFLUORIDE AS A MOBILE PHASE FOR POLAR AND NON-POLAR COMPOUNDS

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SUMMARY

The limits in the elution of non-polar and polar compounds with a supercritical sulfur hexafluoride mobile phase are determined. The compatibility of an SF_6 mobile phase with flame ionization detection during pressure programming in capillary supercritical-fluid chromatography is demonstrated. Special modifications to the split injection assembly are described. A decrease in solvent strength with increasing pressure reported in the literature was not observed. As has been indicated in the literature. SF_6 was found to exhibit low solvating power. Normal alkanes up to about $n = 30$ and polycyclic aromatic hydrocarbons of up to four rings were eluted at 100°C, but no larger compounds of either type. SF_6 has an advantage over CO_2 in that primary amines can be eluted, but it is also limited in most cases to mono-functional amines, and also to mono-functional alcohols and carboxylic acids. No adverse effects on the stationary phase or column deactivation were observed.

INTRODUCTION

Carbon dioxide is the most widely used mobile phase in supercritical-fluid chromatography (SFC). However, elution of primary aliphatic amines is not possible due to formation of insoluble salts. Ammonia has been demonstrated as a viable mobile phase[1,2] and is clearly compatible with amine compounds, but instrumental difficulties have limited its use. Research into mixed mobile phases may yield carbon dioxide-based fluids compatible with primary amines. For instance, methylamine has been used as a modifier, together with water and methanol, in carbon dioxide for the analysis of basic alkaloids[3]. It would be preferable to operate with a neat mobile phase, especially one with a low critical temperature so as to retain the possibility for analysis of thermally labile samples. Several possible mobile phases are short-chain alkanes, halocarbons, or sulfur hexafluoride. SF_6 is the only fluid among these that is compatible with flame ionization detection (FID), which provides a simple and universal detection system.

A comparison of SF_6 to other supercritical-fluid solvents[4], based on solvochromatic data, indicated that SF_6 was weakly polar yet still polarizable and not as

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strong a solvent as CO_2. Schwartz and Brownlee[5] exploited the low solvent strength of SF_6 to effect chemical class separations of hydrocarbon samples with detection via a modified FID system. N,N-Dimethylformamide was used as the injection solution solvent for a series of *n*-alkanes up to hexadecane, but it did not elute from the silica column within 20 min at 50°C and 68 bar. Hellgeth *et al.*[6] used the retention of benzene and alkyl-substituted benzene compounds in packed-column SFC to compare the solvent strength of CO_2 to SF_6. They concluded that SF_6 was a weakly solvating mobile phase and predicted that, in general, only mono-functional polar compounds could be eluted with an SF_6 mobile phase.

In this study the compatibility of an SF_6 mobile phase with FID was further evaluated under capillary column SFC conditions using pressure programming. Unusual problems encountered in split injection operation are discussed and solutions are described. The limits of SF_6 as a mobile phase for non-polar and polar aliphatic and aromatic compounds are determined, with emphasis on the possibilities for elution of amine compounds.

EXPERIMENTAL

The chromatographic system was constructed in our laboratories, and has been described in detail elsewhere[2]. It consisted of a high-pressure syringe pump (Model 8500, Varian Instruments, Walnut Creek, CA, U.S.A.), a gas chromatograph oven (Model 4100, Carlo Erba, Milan, Italy), and an electrically-acuated injection valve (Model C14W, Valco Instruments, Zug, Switzerland) mounted above the oven. The injection loop volume was 0.2 μl. Pressure was controlled from a portable computer (Model HP 75C, Hewlett-Packard, Basle, Switzerland) interfaced to the pump. The pump cylinder was equipped with a cooling jacket, through which a water–ethylene glycol solution was continuously circulated (6°C). Similar to the procedure described by Schwartz and Bronwlee[5], the FID collector assembly was protected from the corrosive action of hydrogen fluoride produced in the flame by plating a 30–50-μm layer of gold over a 0.5-μm layer of nickel (W. Fluehmann, Duebendorf, Switzerland). The effluent gases from the flame ionization detector were drawn through a vacuum scrubber (Model 412, Buchi Laboratoriums-Technik, Flawil, Switzerland) for neutralization in alkaline solution.

The chromatographic column was 5 m × 100 μm I.D. deactivated fused-silica coated with a 0.5-μm film of a 100% methylpolysiloxane (SB-methyl-100, Lee Scientific, Salt Lake City, UT, U.S.A.). The column was connected to an 8 cm × 10 μm I.D. fused-silica restrictor (Infochroma, Zug, Switzerland) via a capillary column union [MVSU/005, Scientific Glass Engineering (SGE), Ringwood, Australia]; a 200-μm I.D. capillary sleeve inside the union served to eliminate dead volume and align the two capillaries. The end of the column restrictor was positioned about 2 mm below the tip of the FID jet, which was operated at 300°C. An auxiliary heating block was attached to the FID collector body and held at 250°C. This was found to eliminate spiking and noise present with either CO_2 or SF_6 mobile phases in this particular instrument (with either standard or gold-plated collectors). No differences in peak symmetry or limits to SF_6 solvating power were observed when a 50-μm I.D. frit retrictor (Lee Scientific) was tested.

Two split injection assemblies were used and are shown schematically in Fig. 1.

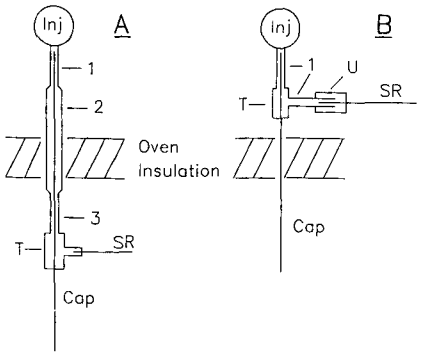

Fig. 1. Schematic diagrams of capillary SFC split injection assemblies (not to scale): (A) design used in previous experiments with 0.5-ml volume, (B) improved design used in this study with 9 μl volume. Inj = injection valve; SR = split restrictor; Cap = capillary column. U = 1/16–1/32 in. Valco reducing union. See text for description of numbered tubing sizes and T-pieces.

The first was our previous design (Fig. 1A), the second (Fig. 1B) was used to produce the chromatograms in this study. In Fig. 1A the T-piece was a 1/16 in. Swagelok union, and the stainless-steel tubing was (1) 15 mm × 0.31 mm I.D. × 1/16 in. O.D., (2) 150 mm × 2.0 mm I.D. × 1/8 in. O.D., and (3) 15 mm × 0.52 mm I.D. × 1/16 in. O.D. In Fig. 1B the T-piece was one-half of a VSU/005 union (SGE), and the tubing pieces (1) were 15 mm × 0.5 mm I.D. × 1/16 in. O.D. The splitter assembly in Fig. 1B is essentially identical to the capillary SFC splitter available from SGE with both tubing pieces reduced in length. The split restrictors were 10 μm I.D. fused-silica, 6 cm long in Fig. 1A and 10 cm long in Fig. 1B. The end of the split restrictor, in the configuration shown in Fig. 1B, was immersed in water to eliminate ice formation which causes flow irregularities.

Liquified SF_6 (SFC-grade, Scott Specialty Gases, Plumsteadville, PA, U.S.A.) was obtained in an aluminium cylinder under its own vapor pressure without an eductor tube, and was used without additional purification. A 2-μm, high flow-rate filter (SS-4FW, Nupro, Lachen, Switzerland) was installed between the cylinder and the pump.

Samples were obtained from Fluka (Buchs, Switzerland). Injection solution concentrations were about 0.5–1 mg ml^{-1} in hexane, or hexane with up to 10% dichloromethane or dichloromethane–methanol as necessary. For retention measurements, the *o*-xylene was dissolved in *n*-pentane. About 100–200 ng of each compound was injected, with some fraction of this amount entering the column. The SF_6 split ratio was 1:12 (column:split) at initial programming conditions of 100°C and 50 bar. The erroneous assumption is sometimes made in capillary SFC studies with split injection that the mobile phase split ratio is also the sample split ratio. The actual amount of sample transferred to the column in this study was not known, and would require an evaluation of the dependence of peak size on split ratio, and other factors, to make a reasonable estimate.

Data were acquired and stored via a 12-bit A/D interface (Nelson Analytical, Cupertino, CA, U.S.A.). The sampling rate was one point per second in all analyses. Chromatograms were replotted such that full-scale corresponds to the equivalent

full-scale response of a strip-chart recorder with the FID electrometer attenuation set at 2^4 (16 pA full scale deflection).

Retention parameter values of *o*-xylene were calculated using its retention time and the time of the first eluting peak of the *n*-pentane solvent (probably a smaller or branched alkane).

RESULTS AND DISCUSSION

The compatibility of an SF_6 mobile phase with FID has been demonstrated for isobaric–isothermal conditions and where the pressure and/or the temperature were step-programmed[5]. The temperature change was from 50 to 100°C, and the pressure change was over the relatively small range of 230 to 340 bar. The packed-column flow-rates were apparently about 50–100 μl min^{-1}, and considering the injection solution concentration and peak size, then the analyses were not performed with the electrometer set at high sensitivity. We sought to determine if SF_6 was compatible under capillary column conditions, where much larger pressure changes are present and the electrometer attenuation is typically set at about 2^4 (16 pA full scale response). FID is selective for C–H bonds, but a mass-flow dependent signal could arise from the presence of hydrocarbon impurities in the SF_6 (*ca.* 5 ppm) or from ionic species formed in the flame. The fluid flow-rate through the capillary column was about 4 μl min^{-1} at initial pressure programming conditions, and was expected to have little influence on the FID signal. The resulting baselines were nearly void of any drift during large pressure changes (50 to 400 bar). This result is demonstrated in the chromatograms discussed below.

Split injection in capillary SFC has been evaluated in a number of laboratories recently with regard to reproducibility and quantitation. With SF_6 as a mobile phase, a problem was encountered that appears to be unique to such a heavy solvent molecule. Experience has shown that "humps" in chromatograms occurring after the solvent peak are usually caused by poor flow paths in the column–restrictor union, such as when the end of either capillary is broken or damaged. Similar humps were observed during initial instrument setup in this study. The humps were not reproducible in size or shape and additional noise and ghost peaks were often observed (Fig. 2). Pressure-programmed chromatograms without an injection produced no peaks, only a baseline with a very slight rise. After several complete and extremely careful assemblies of the capillary union failed to solve the problem, attention was directed to the injection system. The injector split assembly was a prototype used in early SFC experiments in our laboratory (Fig. 1A). The total volume was about 0.5 ml. With a split flow of about 40 μl min^{-1} fluid, 12 min were needed to flush the entire splitter volume. It was also noted that the density of SF_6 (*ca.* 1.6–1.7 g ml^{-1} at 50 bar) is substantially greater than the solvents being used for sample solutions: hexane, dichloromethane, and methanol. There seemed to exist the possibility that, with the large splitter volume and potential for partial miscibility, portions of the injected solvent might not be completely flushed out of the splitter and subsequently enter the column producing the humps and ghost peaks. This theory was not tested with phase equilibria studies; instead, the splitter was exchanged for one with a much smaller volume (Fig. 1B). This eliminated the humps and ghost peaks. Occasionally, some irregularities in the solvent peak shape still arise, which indicates further refinement of

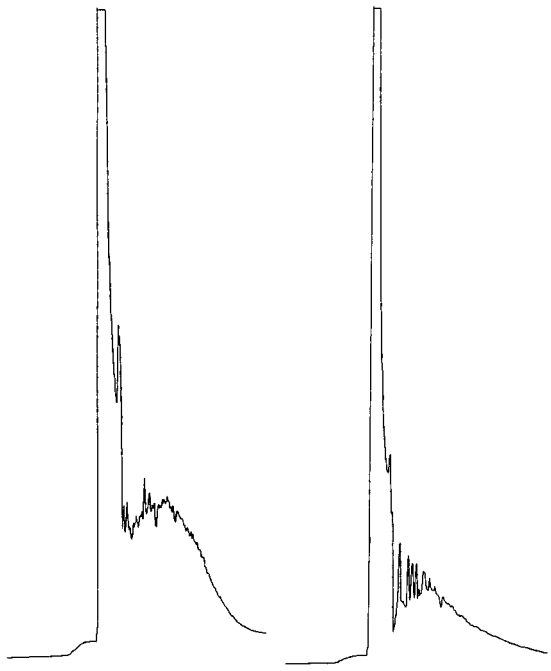

Fig. 2. Examples of unacceptable solvent peaks obtained using the split injection assembly in Fig. 1A.

the splitter assembly may be necessary. For example, most splitters are constructed of 0.5 mm I.D. tubing, which is significantly wider than the capillary SFC column used in this study (*ca.* 200 μm O.D., although wider columns are commercially available). The result is that the capillary column invariably leans against the inside wall of the splitter and is not centered in the flow path from the injection valve as would be optimal. Immiscibility of the sample solvent with the mobile phase can also cause problems. For example, when the sample solvent was about 20% or more methanol, the solvent peak was up to three times as wide as with neat hexane or dichloromethane and often interfered with detection of early eluting peaks.

At 95°C, the retention time of *o*-xylene increased over the pressure range 250–310 bar in a phenyl packed-column SFC system[6]. This increase was suggested to show a concurrent decrease in solvent strength, resulting from decreased mass transfer rates of the solute from the stationary to the mobile phase. The decreased mass transfer rates were attributed to slower solute diffusivity in the mobile phase, which is a result of increased density and viscosity. The pressure–density isotherm of SF_6 at 95°C is shown in Fig. 3.

If the solvent strength decreases under these conditions, then a decrease in retention should also be present in capillary SFC. The retention parameters of *o*-xylene at 95°C over the pressure range 50–350 bar (Table I and Fig. 4) obtained in our capillary SFC system show continuously increasing solvent strength. All retention parameters decrease, and the mobile phase linear velocity increases. The relatively constant capacity factor (k') values at 200–350 bar probably reflect the fact that

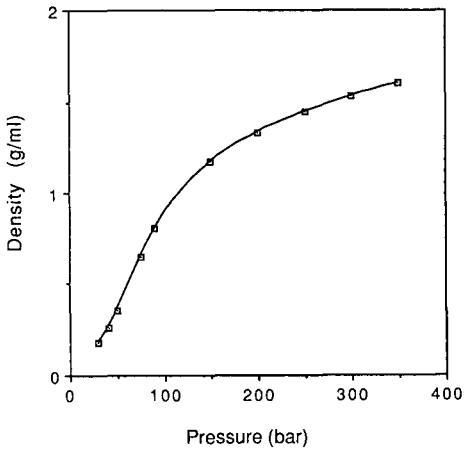

Fig. 3. Pressure–density isotherm of SF_6 at 95°C, calculated from the Peng–Robinson equation of state[7]. Solid line added for clarity.

o-xylene still has a slight affinity for the stationary phase, even though the peak elutes during the last part of the solvent peak at 200 bar and is only a barely detectable shoulder at 350 bar. At such low retention as $k' = 0.08$, the surface of the stationary phase is in contact with a mixture of solvent and mobile phase, disregarding possible swelling and uptake of solvent or mobile phase. Thus, the solute retention is not solely affected by the mobile and stationary phases. Even so, the adjusted retention time (t'_R) values in Table I show continuously decreasing retention, indicative of increasing solvent strength. Since retention time only indicates the position of a peak in a chromatogram, the effect observed by Hellgeth *et al.*[6] was not indicative of a decrease in solvent strength. The effect could have been a consequence of decreased flow-rates (not specified in ref. 6) arising from the increased viscosity and density, or of a lack of thermal equilibrium as has been reported with a carbon dioxide mobile phase[8].

TABLE I

RETENTION PARAMETERS OF *o*-XYLENE IN CAPILLARY SFC AT 95°C

Parameters are: retention time of unretained solute (t_0), *o*-xylene retention time (t_R), adjusted retention time ($t'_R = t_R - t_0$), capacity factor ($k' = t'_R/t_0$), and mobile phase linear velocity (u).

Pressure (bar)	t_0 *(min)*	t_R *(min)*	t'_R *(min)*	k'	u *(cm s^{-1})*
50	11.77	18.77	7.00	0.59	0.71
75	11.00	13.19	2.19	0.20	0.76
90	10.23	11.64	1.41	0.14	0.81
150	6.82	7.44	0.62	0.091	1.22
200	5.31	5.75	0.44	0.083	1.57
250	4.41	4.77	0.36	0.082	1.89
300	3.81	4.13	0.32	0.084	2.19
350	3.40	3.68	0.28	0.082	2.45

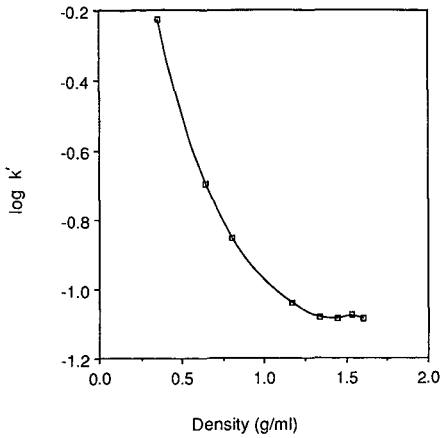

Fig. 4. Retention of *o*-xylene *vs.* SF_6 density at 95°C, densities calculated as in Fig. 3.

In the same study[6], at 224 bar, the retention time of *o*-xylene decreased over the temperature range of 50 to 75°C. This effect was also used to support the theory of decreased solvent strength of SF_6, although the effect of increased solute volatility was also noted to be a factor. Under these same conditions, the retention parameters of *o*-xylene in CSFC (Table II and Fig. 5) indicate that the solute volatility over-compensates for the decrease in density occurring as the temperature increases at constant pressure (Fig. 6).

A mixture of normal aliphatic hydrocarbons was chromatographed (Fig. 7). The alkanes up to $n = 30$–32 were eluted, but the longer alkanes of $n = 36$ and 40 did not elute. This limit in chain length is considerably lower than that found with a CO_2 mobile phase. It is possible that alkanes longer than $n = 30$–32 might elute under different conditions, at a higher temperature for example.

A mixture of four polycyclic aromatic hydrocarbons (PAHs) was injected and pressure programmed at 10 bar min^{-1} from 50 to 400 bar at 100°C. Naphthalene (128 g mol^{-1}), phenanthrene (178 g mol^{-1}), and chrysene (228 g mol^{-1}) eluted, although the chrysene peak was small and broad. Coronene (300 g mol^{-1}) did not elute under these conditions. The limit in molecular weight for PAHs is then about 250 g mol^{-1},

TABLE II

RETENTION PARAMETERS OF *o*-XYLENE IN CAPILLARY SFC AT 224 BAR

Temperature (°C)	t_0 *(min)*	t_R *(min)*	t'_R *(min)*	k'	u *(cm s^{-1})*
50	5.60	6.31	0.71	0.127	1.49
60	5.48	6.05	0.58	0.105	1.52
70	5.29	5.81	0.52	0.098	1.58
80	5.11	5.58	0.47	0.092	1.63
95[a]	4.86	5.26	0.40	0.082	1.72

[a] Values interpolated from data in Table I.

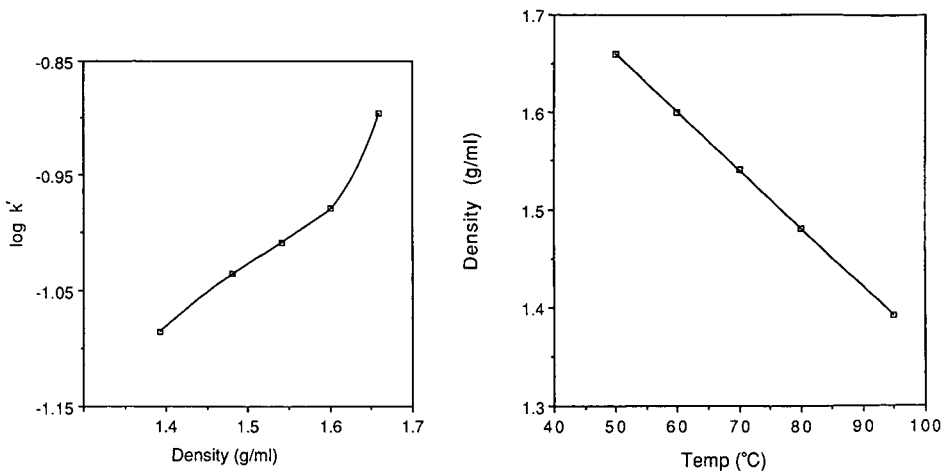

Fig. 5. Retention of *o*-xylene *vs.* SF_6 density at 224 bar, densities calculated as in Fig. 3.

Fig. 6. Temperature–density isobar of SF_6 at 224 bar, calculated as in Fig. 3.

corresponding to compounds such as perylene or benzo[*a*]pyrene. 1,2,4-Trichlorobenzene (TCB) was added to this sample solution (hexane and dichloromethane) to dissolve the coronene. TCB eluted between naphthalene and phenanthrene. The limitations in the elution of aliphatic and aromatic hydrocarbons with an SF_6 mobile

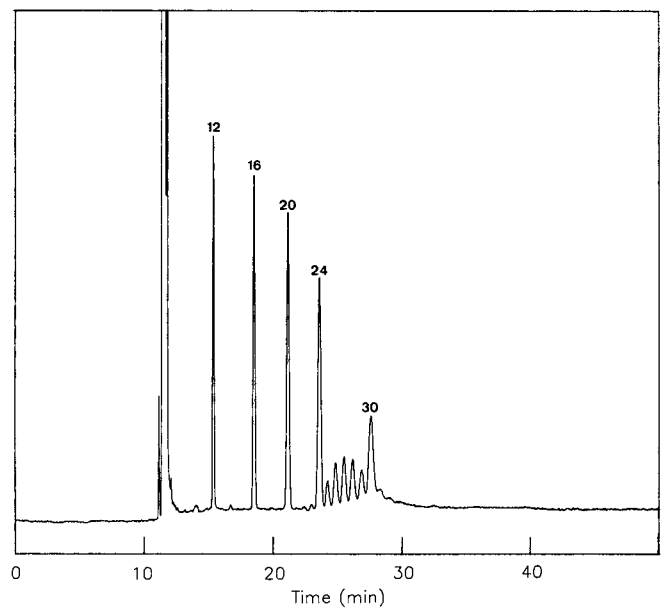

Fig. 7. Capillary SFC chromatogram of a mixture of *n*-alkanes, peak numbers represent alkyl chain length. Conditions: 100°C, pressure programmed at 10 bar min^{-1} from 50 to 400 bar after 10 min inital-pressure hold time, column described in text.

phase imply that application to hydrocarbons group analysis[5] is likewise limited to samples of fairly low molecular weight.

Very little data exist to evaluate the solvating power of SF_6 towards polar compounds. Several polar compounds were injected to assess the limits of SF_6 solvating power for polar compounds. Mono-, di-, and, in some cases, poly-substituted compounds were used. The major functional groups included were hydroxyl, carboxylic acid, and amine, and a few compounds were of mixed functionality. Most of these test compounds can be easily eluted by gas chromatography, and they are used in this study as simple probes for the determination of the properties of an SF_6 mobile phase in capillary SFC.

Considering that benzoic acid[9] is soluble in SF_6, it was expected that aliphatic alcohols (Fig. 8) and a carboxylic acid would elute (Fig. 9). The carboxylic acid peak shows overloading typical on a methylpolysiloxane stationary phase. No information was available as to the solubility of amines in SF_6. Two aliphatic amines were eluted with virtually no peak tailing (Fig. 10). This is the first report of the elution of primary aliphatic amines by SFC with FID. Short-term retention time reproducibility of these amines was excellent, about 0.03% relative standard deviation. Another primary amine which also contains a tertiary amine function (Fig. 11) eluted close to the solvent peak. N-Methylethanolamine eluted even earlier at 12 min under the same programming conditions, also with symmetric peak shape. The aromatic diamine compound *m*-diaminobenzene eluted with symmetric peak shape, but the necessity of adding a signficant amount of methanol to effect dissolution in the injection solution resulted in poor solvent peak shape. In this case the FID signal did not completely return to baseline (*ca.* 10% offset) until 3 min after the end of the solvent peak and at

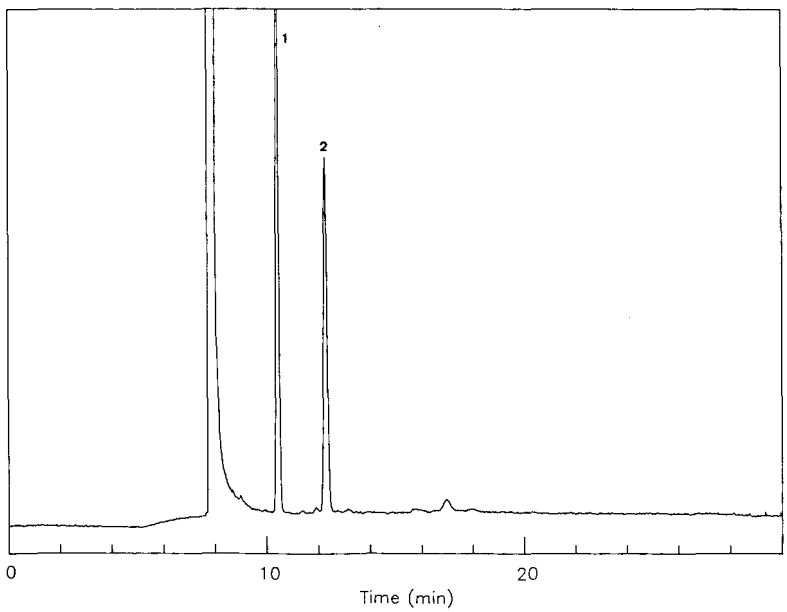

Fig. 8. Capillary SFC chromatogram of (1) 1-decanol and (2) 1-tetradecanol. Conditions: 100°C, pressure programmed from 50 to 300 bar at 20 bar min^{-1} after 5 min at initial pressure.

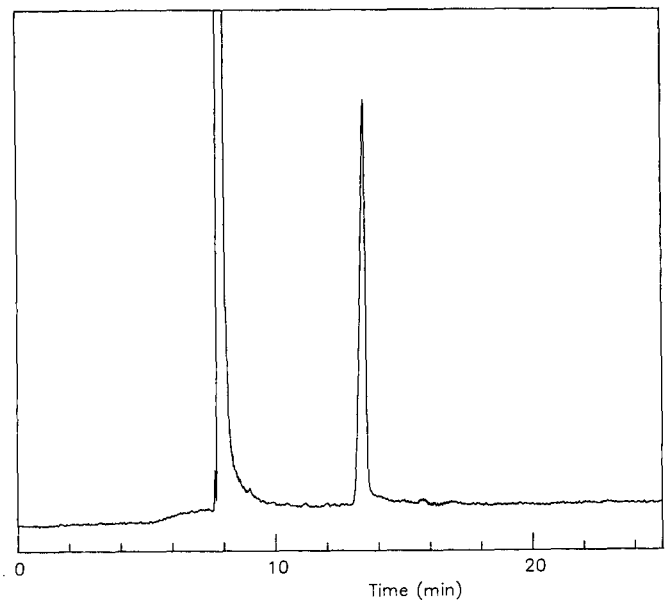

Fig. 9. Capillary SFC chromatogram of tetradecanoic acid. Conditions: same as in Fig. 8.

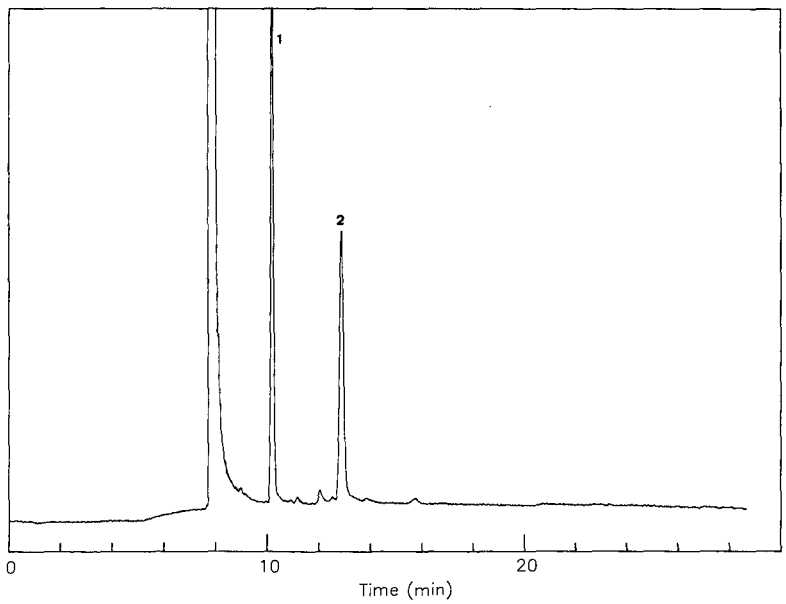

Fig. 10. Capillary SFC chromatogram of (1) 1-aminodecane and (2) 1-aminohexadecane. Conditions: same as in Fig. 8.

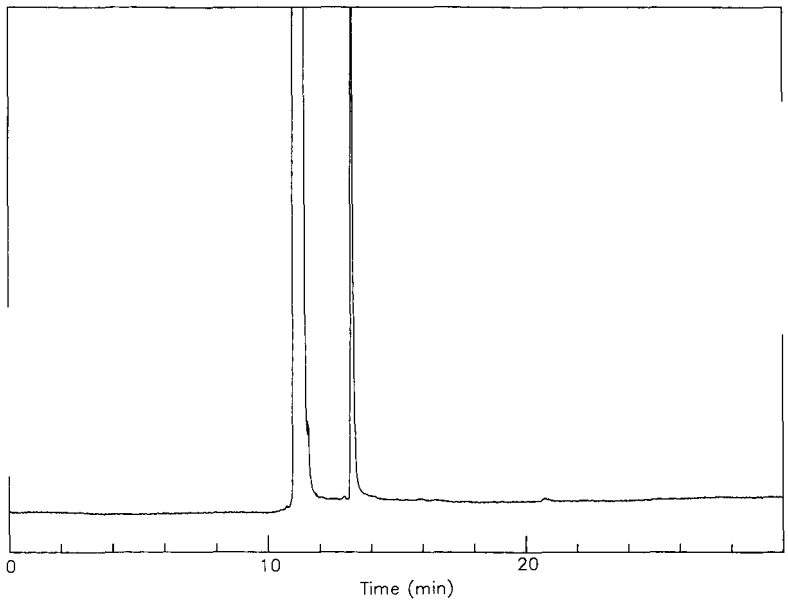

Fig. 11. Capillary SFC chromatogram of 3-diethylamino-1-propylamine. Conditions: same as in Fig. 8, except for 10 min inital-pressure hold time.

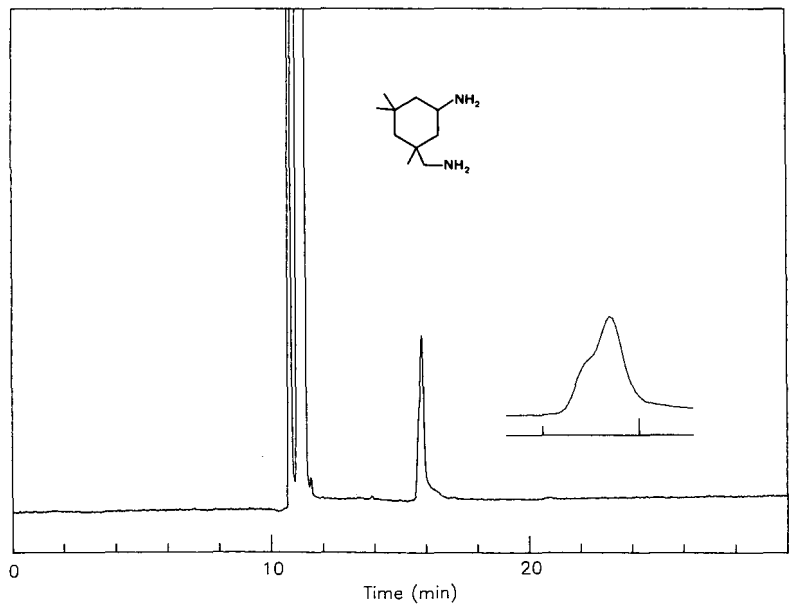

Fig. 12. Capillary SFC chromatogram of isophoron diamine, inset is expanded view. Conditions: same as in Fig. 11.

that point there was a rapid drop in the signal to baseline. This could indicate, together with the injection phenomenon noted above, that there is only limited miscibility of methanol in SF_6. This would hinder the use of methanol as a modifier in SF_6, although other alcohols may be more miscible. Isophoron diamine, an aliphatic diamine, eluted with slight peak tailing (Fig. 12). It was also barely separated into the *cis* and *trans* isomers as shown by the expanded area in Fig. 12. N-Methylethylenediamine apparently eluted but this could not be confirmed due to proximity to the solvent peak. Several other compounds were also eluted with excellent peak shape but are not shown here: anthraquinone, dioctylamine, and several trialkyl amines.

The following polar compounds, injected and pressure programmed at 10 bar min^{-1} from 50 to 400 bar at 100°C, were not eluted by SF_6 capillary SFC: 1,10-decanediol, 1,6-diaminohexane, 1,12-diaminododecane, diethylenetriamine, 4,4′-diaminodiphenylmethane, caffeine, and *p*-aminobenzoic acid. Since *m*-diaminobenzene eluted, it was surprising that 4,4′-diaminodiphenylmethane did not elute. It was assumed that since decanediol did not elute that a similar dicarboxylic acid also would not elute. The prediction by Hellgeth *et al.*[6] that SF_6 would be limited to only mono-functional polar compounds appears to be generally valid.

The column surface deactivation is critical to elution of polar compounds. Initially, an older column was used during the instrument setup. Injection of the aliphatic alcohols produced peaks wih some tailing, while no peaks were seen for injection of the aliphatic amines or acid. The column was replaced by one which was highly deactivated and the results described in this study were obtained. After three weeks of continuous use, the column was tested by gas chromatography for activity and was found to produce almost no tailing for diols or aliphatic amines, similar to the peak shapes initially reported by Woolley *et al.*[10]. SF_6 does not degrade this stationary phase or adversely affect column activity. It is probable that other common stationary phases are also resistant but should be tested.

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CHROM. 21 419

CONTROLE DE CERTAINS SYSTEMES DE CHROMATOGRAPHIE LIQUIDE HAUTE PERFORMANCE À L'AIDE D'UN DÉTECTEUR ÉVAPORATIF À DIFFUSION DE LUMIÈRE

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SUMMARY

Control of some high-performance liquid chromatographic systems by using an evaporative light scattering detector

The quasi universal character of the light scattering detection of microparticles permits in the case of sugar analysis to choose the column and to control its evolution as a function of time. A simple test is proposed to induce the beginning of the regeneration of resinous Ca^{2+}. LiChrosorb Diol support is preferred to aminopropyl silica to realize at the same time a gradient elution and a good sensitivity (30–50 ng).

INTRODUCTION

Les qualités premières exigées d'une colonne de chromatographie liquide haute performance (CLHP), sont la sélectivité et l'efficacité. Deux autres caractéristiques sont cependant nécessaires: une inertie chimique vis à vis des composés et une durée de vie assez longue.

Lorsque certains solutés réagissent avec la phase stationnaire, l'analyse quantitative est perturbée et la durée de vie de la colonne diminuée. C'est le cas des silices greffées aminopropyles, formant une base de Schiff avec le groupe carbonyle des sucres réducteurs[1]. Karlesky *et al.*[2] a, dans ce cas, proposé une méthode pour régénérer les groupes amino de la phase stationnaire.

Généralement, une évolution lente de la phase stationnaire peut entraîner un changement des temps de rétention[3], une modification de la nature du soluté (isomérisation par exemple) ou une adsorption irréversible. Les phases aminopropyles ont été signalées par plusieurs auteurs, comme ayant une durée de vie plus courte que les autres phases greffées[4–6]: la rétention des solutés diminue, ce qui oblige de modifier l'éluant: une augmentation du taux d'acétonitrile ou une addition d'amine peuvent être réalisées dans le cas d'analyse des sucres. Leur dégradation peut provenir en partie d'une certaine instabilité en milieu aqueux qui d'ailleurs apparaîtrait moins avec les greffages provenant de silanes trifonctionnels qu'avec ceux de silanes monofonctionnels[6].

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Cette transformation de la colonne est rarement brutale, et il n'est pas facile d'en déceler le début.

L'objet de cet article est de montrer qu'un choix non arbitraire de supports utilisés pour l'analyse des sucres ainsi que le maintien des performances des systèmes chromatographiques peuvent être réalisés si l'on utilise une détection particulière: la détection de la lumière diffusée par des microparticules résiduelles après évaporation des phases éluantes.

PARTIE EXPÉRIMENTALE

Chromatographie

L'équipement est constitué d'une pompe à gradient Bruker, d'une vanne injecteur Rheodyne, modèle 7125, d'un four Knauer et d'un détecteur évaporatif à diffusion de lumière modèles DDL 10 et DDL 11. Le prototype de ce détecteur a été construit au laboratoire, sous Licence ANVAR[7,8].

L'effluent est nébulisé sous un courant de gaz inerte et le brouillard ainsi formé pénètre dans un tube évaporateur chauffé où le solvant est évaporé. Les microparticules non volatiles de solutés passent à travers le faisceau lumineux d'une lampe source et la lumière diffusée est recueillie à 120° sur un photomultiplicateur. Le modèle DDL 11 vendu par Cunow (Paris) a une sensibilité (30 ng de glucose) meilleure que celle des autres détecteurs du même type. C'est aussi le seul détecteur évaporatif à diffusion de lumière qui permette d'analyser des composés avec l'eau comme éluant et avec une température d'évaporation aussi basse que 45°C, ce qui évite la dégradation thermique des solutés[8].

Colonnes

Les colonnes employées sont les suivantes: Sugar Pak 300 × 6,5 mm, Waters CN 150 × 4,6 mm, Waters Carbohydrate 250 × 4,6 mm (Waters), Zorbax NH_2, Zorbax TMS, Zorbax ODS 250 × 4,6 mm, Zorbax OH 250 × 6 mm (DuPont), LiChrosorb Diol 250 × 7 mm, Lichrospher Diol 250 × 10 mm, LiChrosorb NH_2 150 × 4,6 mm (Merck), R Sil NH_2 Carbohydrate I 250 × 4,6 mm (Alltech), Nucleosil OH 250 × 4,6 mm, Nucleosil NH_2 250 × 4,6 mm (Macherey Nagel), Polypore H 30 × 4,6 mm, Polypore Ca 220 × 4,6 mm (Brownlee Labs.).

Phase mobile

L'eau distillée (Coopération Pharmaceutique Française) et l'acétonitrile RPE ACS (Carlo Erba) étaient utilisés comme phase mobile. Il faut signaler qu'un bruit de fond assez important était observé avec certaines qualités d'acétonitrile. Notons que ce bruit n'est pas en relation directe avec le taux de résidu sec et nous ne pouvons pas, à l'heure actuelle, expliquer ce phénomène. Nous conseillons donc, pour vérifier la qualité du solvant, de brancher directement le détecteur sans colonne: la ligne de base doit être identique à celle observée lorsque la pompe est arrêtée.

Absorption atomique

La quantité de silice et de calcium dans l'effluent est déterminée par absorption atomique avec un appareil Perkin Elmer, modèle 3030.

pH mètre

La mesure en continu du pH en sortie de colonne est réalisée par un détecteur pH métrique Sensorex (Prolabo).

RÉSULTATS ET DISCUSSION

L'analyse des sucres par chromatographie liquide peut être réalisée en utilisant les résines échangeuses d'ions Ca^{2+} ou les silices greffées aminopropyles ou octadécyles, les différents systèmes offrant des sélectivités différentes et complémentaires[8]. Sur résines calcium, il est possible d'éluer rapidement les polysaccharides alors que ces derniers ne sont pas élués sur silices aminopropyles avec les teneurs en eau habituelles (20–50%), ni sur silices octadécyles sans l'apport de méthanol à l'éluant aqueux[8].

Nous choisirons comme système de référence une colonne de silice greffée octadécyle avec de l'eau pure comme éluant. Dans ces conditions, l'effluent donne avec le DDL 11 une quantité de lumière diffusée pratiquement égale à celle obtenue lorsque la pompe est arrêtée. Ceci indique qu'en sortie de colonne, il n'y a aucune entité non évaporable.

Étude des resines echangeuses d'ions Ca^{2+}

Les sucres et les polyols sont analysés avec une bonne sélectivité sur résines Ca^{2+} avec l'eau comme éluant à 80–90°C. Quand la température de la colonne est à 40–60°C, l'effluent aqueux entraîne un signal similaire à celui obtenu avec l'effluent d'une silice greffée octadécyle. Par contre, lorsque cette température augmente jusqu'à 80–90°C, la quantité de lumière diffusée augmente et un bruit de fond plus important que précédemment apparait[7]. Cela correspond à la sortie de sels de Ca^{2+}, ce qui a été vérifié par absorpton atomique. Malgré tout, ce bruit de fond est très régulier. De plus, lors de leur détection, les sucres complexés au calcium engendrent un signal supérieur à celui obtenu en sortie de colonne ODS avec l'eau pure (Fig. 1). Il en résulte un rapport signal sur bruit avec ces mêmes sucres, voisin de celui obtenu sur silice ODS.

Au bout d'un certain temps d'utilisation, les sites SO_3^- du support ayant perdu le co-ion Ca^{2+} deviennent acides et dégradent les sucres rapidement à cette température élevée: la Fig. 2 montre que le saccharose injecté génère *in situ* du glucose et du fructose (Fig. 2b et c), laissant croire à la présence de ces deux monosaccharides dans l'échantillon de saccharose. L'observation de la forme de ces deux pics montre qu'ils n'ont pas la finesse de ceux de glucose et du fructose lorsqu'ils sont injectés avec le saccharose.

Comme le détecteur DDL 11 est sensible, il permet de connaître l'état de la colonne en injectant une solution fraîche de saccharose et en notant l'apparition des pics de glucose et de fructose. Si cela se produit, il convient alors de régénérer la colonne en percolant une solution de nitrate ou d'acétate de calcium. L'injection de saccharose permet de contrôler alors que la régénération a été efficace.

Il faut noter que cette hydrolyse des sucres générée *in situ* sur les sites acides, n'est notable que la température est à 80–90°C: des essais réalisés sur une colonne Ca^{2+} neuve, thermostatée à 80°C, et précédée d'une précolonne remplie de résines H^+ maintenue à 20°C, ont montré qu'il n'y a pas d'hydrolyse du saccharose (Fig. 2d). Au contraire, si la précolonne et la colonne sont chauffées à 80°C, il y a hydrolyse rapide *in situ* du saccharose (Fig. 2e).

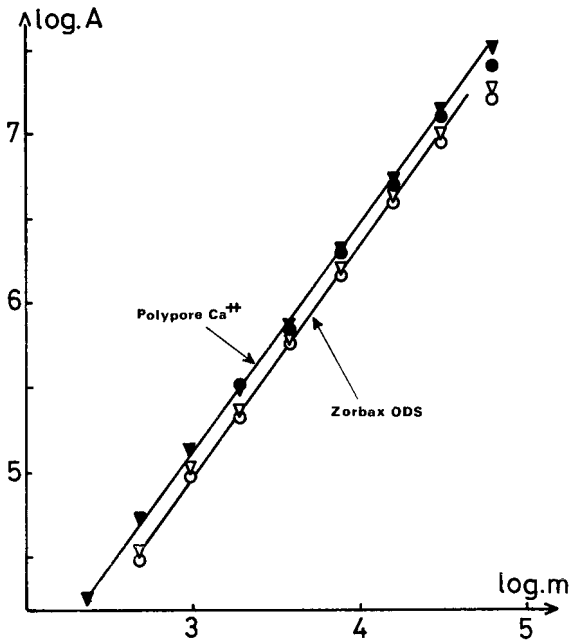

Fig. 1. Courbe de calibration aire (A) du pic en fonction de la quantité injectée (m) en ng pour le glucose (▽, ▼) et le raffinose (○, ●). Colonnes Polypore Ca^{2+} et Zorbax ODS. Eluant: eau. Détecteur DDL 10. Température du tube évaporateur, 45°C. Pression du gaz nébuliseur, 2,2 bars.

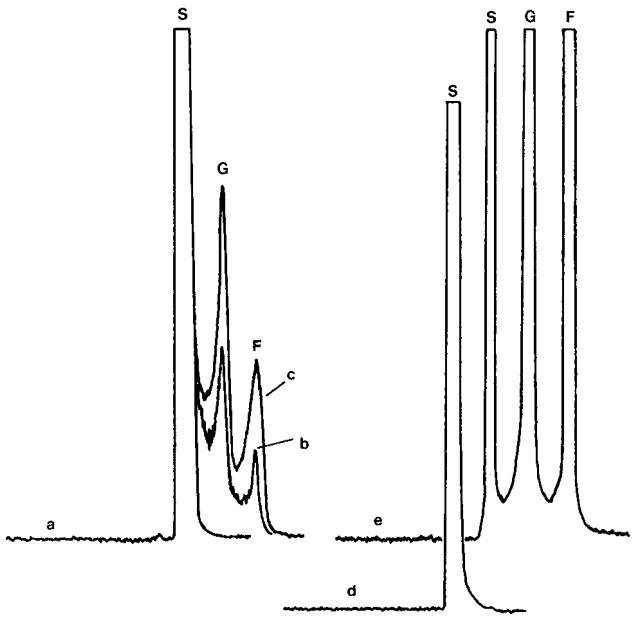

Fig. 2. Analyse du saccharose (S) (28 g/l) sur colonne Sugar Pak. Eluant: eau 80°C. Débit: 0,4 ml/min. (a) Sugar Pak neuve: sans précolonne. (b et c) Sugar Pak usagée: apparition des pics correspondant au glucose (G) et fructose (F) générés *in situ*. (d) Sugar Pak neuve à 80°C avec précolonne Polypore H à 20°C. (e) Sugar Pak neuve et précolonne Polypore H à 80°C. Détecteur DDL 11 réglages voir Fig. 1.

Étude des supports siliceux aminopropyles

C'est le type de support le plus utilisé pour l'analyse des sucres, mais c'est aussi celui que l'on a très vite signalé comme ayant une durée de vie courte: on observe un tassement du support avec apparition de pics dédoublés, occasionnés par un volume mort en tête de colonne.

Comme le détecteur DDL permet de réaliser des gradients acétonitrile-eau, sans dérive de ligne de base[7], l'analyse de mélanges complexes de sucres (mono-, di-, polysaccharide) devrait être réalisable avec de telle colonne en augmentant la quantité d'eau dans l'éluant.

Le profil d'un tel gradient est montré Fig. 3. On note que lorsque le pourcentage d'eau atteint 30–40%, la ligne de base dérive et le bruit de fond augmente, rendant toute analyse impossible á haute sensibilité. La dérive de la ligne de base correspond á une quantité de lumière diffusée croissante provenant de microparticules non évaporables. Le bruit de fond plus important indique que la quantité de microparticules qui diffusent n'est pas constante même à court terme. Avec un éluant isocratique acétonitrile–eau (65:35), la sensibilité du DDL 11 est limitée à 1 ug environ. Plusieurs supports commerciaux aminopropyles ont été ainsi comparés et tous

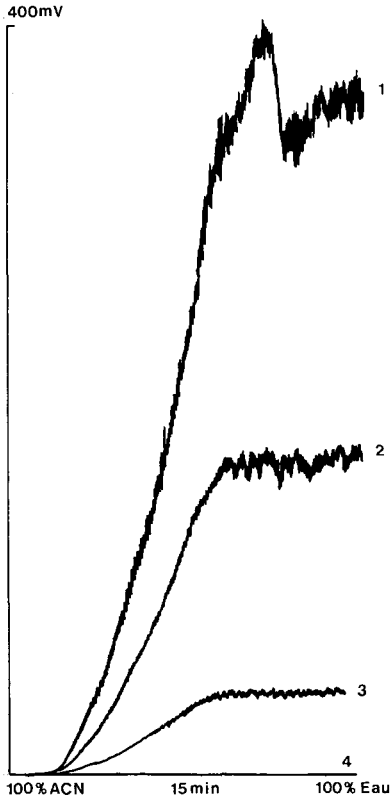

Fig. 3. Dérive de la ligne de base avec les colonnes de silices greffées aminopropyles lors d'un gradient acétonitrile 100% à eau 100%. Débit: 2 ml/min. (1) Lichrosorb NH_2; (2) Nucléosil OH; (3) Zorbax OH; (4) LiChrosorb Diol, Zorbax ODS, Lichrospher Diol. Détecteur DDL 11 réglages voir Fig. 1.

donnent la même caractéristique: les microparticules restantes après l'évaporation de l'éluant créent un bruit de fond important. Pour connaître la nature de ces microparticules, l'effluent des colonnes aminopropyles, avec comme éluant l'eau pure, a été analysé par absorption atomique (Tableau I). En moyenne, l'effluent contient une teneur de 30 ppm en silicium, alors que celui provenant d'une colonne contenant de la silice Zorbax Sil, a une teneur inférieure à 1 ppm, comme dans le cas d'un support greffé apolaire ODS ou TMS (Tableau I).

Cette hydrolyse des supports greffés aminopropyles permet d'expliquer le tassement des colonnes correspondantes. Par contre, la très faible hydrolyse relative de la silice laisse penser que le silicium se trouvant en solution dans l'effluent doit provenir principalement du greffon. Cette coupure probable du greffon n'étant pas un phénomène régulier pourrait expliquer le bruit de fond important contrairement à ce que l'on observe en sortie des colonnes échangeuses d'ions calcium où le départ du calcium du support est un phénomène régulier. Cette hydrolyse doit d'ailleurs limiter également la sensibilité du détecteur réfractométrique, car l'indice de réfraction de l'éluant, même avec thermostatation, doit dépendre du taux de silicium partant en solution à court terme.

Puisqu'il y a dissolution du support aminopropyle dans l'eau, nous avons voulu voir l'influence du pH et de la force ionique de l'éluant sur cette dissolution. La mesure du pH de l'effluent a été réalisée (Tableaux I et II). Si l'eau distillée à pH 6,3 est percolée à travers la colonne aminopropyle, on note en sortie un pH de 9, sur Zorbax NH_2 et Nucléosil NH_2, alors qu'au contraire il est de 4 sur RSil NH_2 et LiChrosorb NH_2 (Tableau I). Il est curieux de constater que les deux supports sphériques Zorbax et Nucléosil entraînent une augmentation du pH, alors que c'est l'inverse pour ceux de forme irrégulière RSil et LiChrosorb. Une distinction d'après le pH en milieux aqueux entre les silices sphériques et irrégulières a déjà été notée par Engelhardt et Müller[9].

TABLEAU I
ANALYSE D'EFFLUENT SUR DIFFÉRENTES COLONNES
Eluant: eau distillée à pH 6,3.

Support	*pH effluent*	*Si (ppm)*
Zorbax Sil		< 1
Zorbax NH_2	8,5–9	38
LiChrosorb NH_2	4,0	41
Nucléosil NH_2	9,0–9,1	
Waters Carbohydrate RSil NH_2		24
Carbohydrate I	4,1	26
Nucléosil OH	4,7	15
LiChrosorb Diol	6,4	< 1
Zorbax OH		< 1
Zorbax ODS	6,1	< 1
Zorbax TMS	6,1	< 1
Waters CN		< 1

TABLEAU II
EFFET DU pH DE L'ÉLUANT AQUEUX SUR COLONNE ZORBAX NH_2
TFA = acide trifluoroacétique; ACN = acétonitrile.

	Additif	*pH de l'éluant*	*pH de l'effluent*
A	–	6,5	8,8
B	TFA 10^{-4} *M* + HCO_3NH_4	7,0	9,2
C	TFA 10^{-5} *M* + HCO_3NH_4	7,0	9,2
D	TFA	5,15	9,4
E	TFA	4,6	9,4
F	ACN–H_2O (80:20)	5,7*	9,0*
G	ACN–H_2O (65:35)	6,15*	9,2*
H	ACN–B (65:35)	8,15*	9,3*

De façon générale, l'étude réalisée sur des supports greffés de nature différente (ODS, TMS, NH_2, OH, CN) montre que l'hydrolyse représentée par la concentration en silice dans l'effluent existe en même temps que l'on note un écart de pH entre l'éluant et l'effluent.

L'utilisation d'un détecteur évaporatif à diffusion de lumière interdit en général l'adjonction à l'éluant de sels qui engendreraient un bruit de fond trop important. Le DDL 11 autorise l'emploi de composés facilement évaporables comme l'acide trifluoroacétique (TFA) et des mélanges TFA + HCO_3NH_4 [10].

Le pH et la force ionique de l'éluant sont modifiés par la présence de ces composés. D'après les résultats du Tableau II obtenus avec le support Zorbax NH_2, on note que quel que soit le pH ou la force ionique de l'éluant, le pH de l'effluent reste de 9 environ, comme en présence de l'eau distillée, ce qui laisse supposer que l'élément en solution provenant de la colonne impose son pH, à la fois par son pKa et sa concentration.

Une mesure en milieu hydroorganique avec l'acétonitrile montre la même augmentation du pH. Dans le Tableau II, la mesure de pH en milieu hydroorganique est notée pH*.

La conclusion de cette étude sur les supports aminopropyles, est que le détecteur évaporatif à diffusion de lumière est un moyen simple pour suivre, comme dans le cas des colonnes Ca^{2+}, l'état de fonctionnement de la colonne. L'augmentation de la dérive de la ligne de base étant proportionnelle à la quantité de silice dans l'effluent.

L'emploi du gradient d'élution acétonitrile-eau avec les silices greffées aminopropyles reste donc délicat, voire impossible, si l'on désire en même temps qu'une analyse performante en sélectivité, une assez bonne sensibilité (30-50 ng).

Étude des silices greffées diol

Plusieurs silices greffées de ce type sont proposées sur le marché et en général pour des analyses de composés autres que les sucres. Elles n'offrent pas l'inconvénient des silices aminopropyles de donner une base de Schiff avec les sucres réducteurs. Leurs greffons, d'après la littérature, sont tous du type $-(CH_2)_3-O-CH_2-CHOH-CH_2OH$ et pourtant leur sélectivité est différente.

Le support Nucléosil OH de Macherey Nagel retient peu les sucres. Il faut donc

employer un éluant (acétonitrile-eau) très faiblement chargé en eau (10%), pour avoir une rétention et une séparation convenables de mono et disaccharides (Fig. 4). Il est alors nécessaire, pour éviter une perte d'efficacité, d'injecter les solutés dans un mélange très riche en acétonitrile, ce qui n'est pas toujours aisé, vu la faible solubilité des sucres en un tel milieu.

Ce support, du fait de la faible rétention des sucres, pourrait convenir pour étudier les polysaccharides, en augmentant la teneur en eau de l'éluant. Ceci doit être fait avec précaution, car l'eau pure hydrolyse ce support (Fig. 3, Tableau I). L'intensité de la lumière diffusée correspond à une perte de silicium plus faible qu'avec les supports aminopropyles. Le signal du bruit de fond obtenu au DDL 11 avec ce support, correspondant à une perte en Si, est malgré tout plus faible qu'avec les supports aminopropyles (15 ppm mesuré par absorption atomique).

Ce support a un autre inconvénient lorsqu'il est utilisé dans l'analyse des sucres. On peut noter une évolution rapide dans le temps de ses performances après un nombre assez faible d'analyses: les chromatogrammes de la Fig. 4 réalisés dans l'ordre du vieillissement de la colonne illustrent cette évolution.

Le chromatogramme I montre une séparation correcte du glucose forme α et du fructose. Après plusieurs analyses, la colonne permet la séparation de mélanges d'anomères α et β de sucres tels que Glucose, Galactose, Lactose, Cellobiose et on peut montrer que ces anomères ne sont pas engendrés au cours de la chromatographie. En effet, une étude a montré que si les sucres sont maintenus dans un solvant organique limitant l'anomérisation tel que le méthanol, la formation d'anomères n'apparaît pas; au contraire on observe les deux pics anomères, si l'on réalise l'anomérisation en milieu aqueux et que l'échantillon est dilué avec de l'acétonitrile (80%), ceci afin d'éviter le dédoublement de pics qui peut se produire chaque fois qu'une trop grande différence entre l'éluant et le solvant d'injection existe. Dans le cas du glucose, on peut même suivre la cinétique de la mutarotation. On en conclut que la colonne Nucléosil OH ne crée pas "*in situ*" les anomères, mais permet leur séparation.

Cette évolution du support présenterait un intérêt si elle était stable, réversible ou reproductible, ce qui n'est pas le cas. Le Chromatogramme III de la Fig. 4 montre la séparation des anomères α et β du galactose et le Chromatogramme IV montre la même

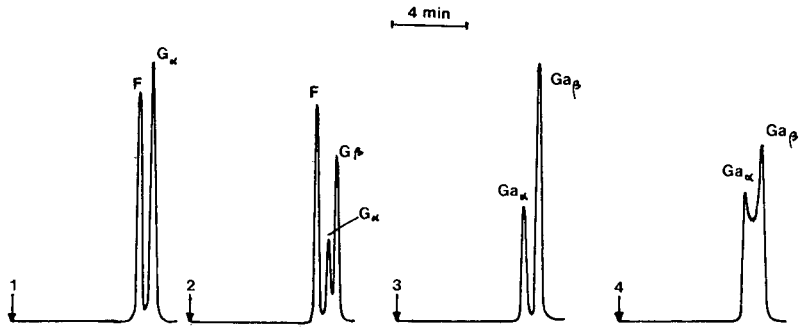

Fig. 4. Evolution au cours du temps d'une colonne Nucléosil OH lors de l'analyse de sucres. (1) Colonne neuve; (2, 3, 4) au cours du viellissement. Colonne: Nucléosil OH 250 × 4,6 mm. Eluant: acétonitrile–eau (90:10). Débit: 0.9 ml/min. F = Fructose, G_α = glucose α, G_β = glucose β, Ga_α = galactose α, Ga_β = galactose β. Détecteur DDL 11 réglages voir Fig. 1.

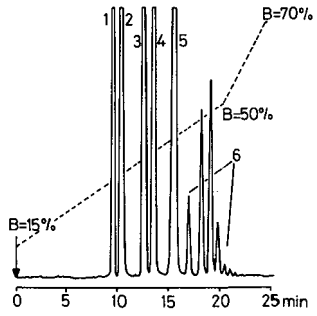

Fig. 5. Analyse d'un mélange de sucres en gradient d'élution. Colonne: Lichrospher Diol, 250 × 7 mm. Eluant: acétonitrile (A), eau (B). 1 = Fructose; 2 = glucose; 3 = saccharose; 4 = lactose; 5 = raffinose; 6 = dextranes. Détecteur DDL 11 réglages voir Fig. 1.

séparation au bout d'un temps plus long d'utilisation. Des essais de régénération n'ont rien donné et il a été impossible de la ramener à son état initial.

Pour des analyses de sucres ce support n'a pas été retenu, de même que le Zorbax OH non sélectif pour les monosaccharides.

En conclusion de cette étude des supports Diol, seuls les LiChrosorb et Lichospher Diol offrent une bonne tenue à l'hydrolyse: pas de dérive de ligne de base en gradient (Fig. 3), pas de dissolution du support (Tableau I), pas de phénomène de séparation d'anomères. Ces supports conduisent à une analyse performante des sucres en gradient d'élution et à une excellente sensibilité avec la diffusion de lumière par microparticules (Fig. 5).

CONCLUSION

La diffusion de lumière par microparticules apparaît non seulement comme un moyen de détection quasi universel mais elle permet en outre de suivre l'état du système chromatographique. L'application au domaine des sucres est illustrée dans ce travail où cette technique offre un moyen de sélection du support chromatographique et un suivi de l'évolution du système.

RÉSUMÉ

Le caractère quasi universel de la détection par diffusion de la lumière par microparticules permet, dans le cas de l'analyse des sucres, de choisir la colonne et de suivre son évolution en fonction du temps. Un test simple est proposé pour prévoir la régénération des résines Ca^{2+}. Le support LiChrosorb Diol est préférable aux silices greffées amino-propyles, si l'on veut réaliser en même temps un gradient d'élution et une bonne sensibilité (30–50 ng).

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SPECIFIC SCREENING METHODS FOR GLUCOSINOLATES IN SPROUT EXTRACTS USING ON-LINE THERMOSPRAY LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

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SUMMARY

Considering the growing importance of the determination and identification of (desulpho-)glucosinolates in extracts of plant materials, rapid screening methods for desulphoglucosinolates were developed, based on thermospray liquid chromatography–mass spectrometry (LC–MS) and LC–MS–MS. In thermospray LC–MS the screening of plant extracts containing various desulphoglucosinolates can be based on the group-specific fragment at m/z 214, but identification of the peaks is not always straightforward owing to the lack of compound-specific fragments in the mass spectra. Using a constant neutral loss of 162 a.m.u., corresponding to the loss of the sugar ring, in thermospray LC–MS–MS the screening and identification of the various desulphoglucosinolates gives better results, especially because the signal in the MS–MS mode is less dependent on the fluctuating thermospray conditions. In some applications direct thermospray MS–MS analysis of mixtures, injected in the flow-injection mode, can give satisfactory results.

INTRODUCTION

Glucosinolates are found naturally in cruciferous plants, such as brassica vegetables, which are important in the human diet. The qualitative and quantitative analysis of glucosinolates (general formula and examples are shown in Table I) has received much attention in recent years[1]. Glucosinolates are of particular interest in food research, because the enzymatically released aglucones are responsable for the flavour and odour of many vegetables, *e.g.*, sprouts[2,3]. A number of them may have adverse effects on health[3].

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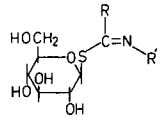
TABLE I

GENERAL STRUCTURES OF GLUCOSINOLATES AND DESULPHOGLUCOSINOLATES AND THE COMPOSITION OF THE R GROUP OF THE COMPOUNDS ANALYZED



R′ = SO_4, glucosinolates; R′ = OH, desulphoglucosinolates.

Desulphoglucosinolate	*Side-chain (R)*	*Mass*	*Molecular weight*
Glucobrassicin	CH_3–	15	253
Sinigrin	$CH_2=CHCH_2$–	41	279
Gluconapin	$CH_2=CH(CH_2)_2$–	55	293
Progoitrin	$CH_2=CHCHOHCH_2$–	71	309
Glucotropaeolin	$C_6H_5CH_2$–	91	329
Glucoiberin	$CH_3SO(CH_2)_3$–	105	343
Gluconasturtiin	$C_6H_5(CH_2)_2$–	105	343
Glucobrassicin	Indole-3-CH_2–	130	368
Neo-glucobrassicin	4-Methoxyindole-CH_2–	160	398

The separation and analysis of glucosinolates can be performed by either gas chromatography (GC) or liquid chromatography (LC), using flame ionization detection and UV detection at 230 nm, respectively. However for GC separation, application of trimethylsilyl derivatization is necessary[4]. During the silylation the glucosinolates also undergo desulphation, so pertrimethylsilyl desulphoglucosinolates are obtained. For direct analysis of glucosinolates, various high-performance liquid chromatography (HPLC) systems have been developed. Separation of glucosinolates can be performed by ion-exchange chromatography[5] or by reversed-phase ion-pair LC[6]. By these methods the glucosinolate fraction can be well separated from the other plant constituents.

A major breakthrough in glucosinolate analysis has been achieved with the introduction of enzymatic on-column desulphation using aryl sulphatase[7]. The enzymatic procedure, which converts the glucosinolates into their desulpho analogues, also acts as a specific clean-up method. Desulphoglucosinolates can be analysed either by GC, after silylation to the pertrimethylsilyl derivatives[8], or by reversed-phase HPLC with gradient elution using acetonitrile–water mixtures as the mobile phase and UV detection[9].

Several mass spectrometric techniques have been investigated for structure elucidation of the various (desulpho-)glucosinolates, *e.g.*, direct probe electron impact[10], chemical ionization[11] and fast atom bombardment[12]. Considerable structural information can be obtained with these techniques.

As the glucosinolates are present in complex mixtures, much attention has also been given to combined chromatography mass spectrometry. Gas chromatography–mass spectrometry (GC–MS) of the pertrimethylsilyl desulpho derivatives has been performed with either electron impact or ammonia chemical ionization. The latter technique appears to provide more useful structural information[13]. Recently, some preliminary experiments on the analysis of underivatized glucosinolates have been

performed in our laboratory, using continuous flow fast atom bombardment mass spectrometry. The results obtained appear to be very promising[14].

After the addition of ammonium acetate, commonly used reversed-phase HPLC mobile phases are suitable for thermospray liquid chromatography–mass spectrometry (TSP LC–MS) in the TSP buffer ionization mode. The desulphoglucosinolates show considerable fragmentation in TSP LC–MS, yielding weakly or even non-detectable protonated molecules, several ions due to the glucosyl and thioglucosyl moieties and ions giving information on specific parts of the molecule[15,16].

There is a need for a rapid and specific screening method for glucosinolates in plant material, *e.g.*, sprout extracts. It should provide detection of desulphoglucosinolates and either confirmation of their identity or identification. This paper describes the investigation of the applicability of TSP LC–MS and TSP LC–MS–MS for this purpose. The results obtained with TSP LC–MS confirm most of the findings of others[15,16], but provides additional information with respect to the use of tandem mass spectrometry (MS–MS). The possibility of analysing sprout extracts directly by TSP MS–MS without chromatographic separation was also investigated.

EXPERIMENTAL

Samples and sample pretreatment

The plant material studied consisted of Brussels sprouts, variety "Roger". The total glucosinolate content, as determined by the glucose-release method[17], was 3800 μmol/kg on a fresh weight basis. The sprouts were extracted with boiling 100% methanol and re-extracted with boiling 70% methanol. After removal of the volatile solvent *in vacuo* the sample was stored at −20°C until required.

An aliquot of the sample was analysed by HPLC with UV detection at 230 nm[9]. Based on retention times, the following glucosinolates were identified (the concentrations found, in μmol/kg on a fresh weight basis, are given in parentheses): glucoiberin (68), progoitrin (414), sinigrin (1832), gluconapin (117), glucobrassicin (1080) and neoglucobrassicin (118). The sum of the six glucosinolates identified amounted to 96% of the total glucosinolate content as determined by the glucose-release method.

Liquid chromatography

The HPLC system, combined with the mass spectrometer, consisted of two Model 2150 high-pressure pumps, a Model 2152 gradient controller (LKB, Bromma, Sweden), a Model 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) and a Spherisorb ODS-2 (5 μm) column (250 × 4.6 mm I.D.) (Tracer Analytica, Barcelona, Spain).

During a gradient run, the amount of eluent B [acetonitrile–water (20:80) containing 50 mmol/l ammonium acetate] in eluent A (50 mmol/l ammonium acetate in water) was increased from 1 to 50% within 11 min, from 50 to 99% in 5 min and kept at 99% for 14 min. The eluent flow-rate was 1.0–1.5 ml/min.

Mass spectrometry

LC–MS and LC–MS–MS experiments were performed using a Finnigan-MAT TSQ 70 triple quadrupole MS–MS system (Finnigan-MAT, San José, CA, U.S.A.), equipped with a Finnigan-MAT TSP interface. The vaporizer temperature and repeller potential were optimized. The block temperature was kept at 200°C.

MS–MS was performed in the daughter and neutral loss scan modes. The collision energy was optimized for the various compounds. Air was used as collision gas with a pressure of 0.05 Pa, measured in the collision quadrupole. Because the pressure in the analyser quadrupole is rather high owing to the TSP conditions in the ion source, the admittance of a collision gas in the collision cell is not always necessary.

RESULTS AND DISCUSSION

Reversed-phase LC using gradient elution with acetonitrile as the organic modifier can be used to separate desulphoglucosinolates in plant extracts[9]. This solvent system can be used for TSP LC–MS when ammonium acetate is added. The chromatographic separation of the desulphoglucosinolates is not adversely influenced. The ions observed in the TSP mass spectra obtained from some standards (*e.g.*, sinigrin) were similar to those observed by others[15,16]. The general appearance of the thermospray LC–MS traces of the desulphoglucosinolates is summarized in Table II and illustrated for glucobrassicin. The assignment of the various fragments must be considered tentative until supporting evidence is forthcoming. Usually a protonated molecule is observed (for some compounds only with low intensity), sometimes accompanied by small sodium cationized molecules. Ammoniated molecules, which were reported by Mellon *et al.*[15], were not found in these experiments. Characteristic peaks related to the (thio-)glucosidic part of the molecule (group-specific fragments), which occur at *m/z* 180, 198, 214 and 240 are explained in Table II. Some compound-specific fragments containing the characteristic R group were also found. A rearrangement peak of general formula $(RN{=}C{=}O + NH_4)^+$ was observed for most of the desulphoglucosinolates studied. Some of the other specific fragments, which have been reported by Hogge *et al.*[16], were only observed in some of the spectra and others were not found at all. Some of the compound-specific fragments appear at relatively low masses, *i.e.*, below *m/z* 150, where the background ions, *e.g.*, solvent cluster ions, are also present with relatively high intensity. This hinders the interpretation of the mass spectra of the desulphoglucosinolates with molecular weights below 330 a.m.u. In principle, identification of the various compounds can be

TABLE II

DIAGNOSTIC FRAGMENTS IN THE TSP MASS SPECTRA OF DESULPHOGLUCOSINOLATES, ILLUSTRATED FOR GLUCOBRASSICIN (FIG. 2)

Group-specific fragments	*m/z*	*Compound-specific fragments*	*m/z*	*Glucobrassicin*
$[C_6H_{10}O_5 + NH_4]^+$[a]	180	$[M + H]^+$		369
$[C_6H_{10}O_5 + H_2O + NH_4]^+$	198	$[M + Na]^+$		391
$[C_6H_{11}O_5SH + NH_4]^+$[a]	214	$[M + K]^+$		407
$[C_6H_{11}O_5SCH{=}NOH + H]^+$	240	R^+[b]		130
		$[RCN + H]^+$	R + 27	157
		$[RCN + NH_4]^+$	R + 44	174
		$[RN{=}C{=}O + NH_4]^+$[a]	R + 60	190
		$[RC(=NOH)SH + H]^+$[b]	R + 77	207

[a] Structure proposed by Mellon *et al.*[15].
[b] Structure proposed by Hogge *et al.*[16].

performed by interpretation of the compound-specific fragments. However, the identification is not always straightforward, as the relative intensities of the various fragment ions in the spectrum are strongly influenced by the TSP experimental conditions, which explains the differences observed compared with other studies. One of the drawbacks of TSP LC–MS is the difficulty of reproducing the ionization conditions. The appearance of the TSP mass spectrum is influenced by several interdependent parameters, such as the repeller potential, the vaporizer temperature, the block temperature, the composition of the mobile phase, the flow-rate, the contamination of the repeller electrode and the performance of the spray. Some of these experimental conditions cannot be reproduced from day to day, *e.g.*, the spray performance and the repeller electrode contamination.

As an illustration of the difficult reproducibility of TSP mass spectra, Fig. 1 shows two mass spectra of progoitrin, obtained under comparable conditions on different days. In Fig. 1B the compound-specific fragments $[M + H]^+$ (m/z 310),

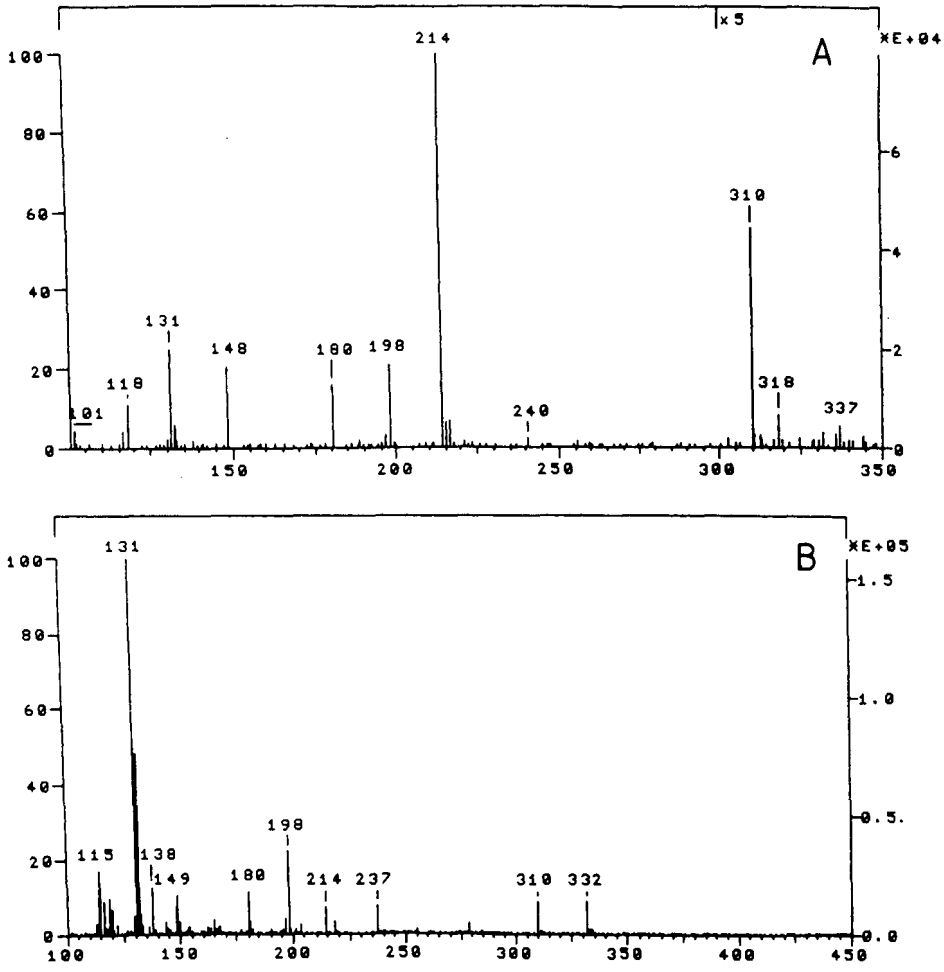


Fig. 1. TSP mass spectra of desulphoprogoitrin, obtained under comparable conditions on different days.

$[M + Na]^+$ (*m/z* 332) and $[RNCO + NH_4]^+$ (*m/z* 131) appear to be more intense than the group-specific fragments at *m/z* 180, 198, 214 and 240, which will make identification of the various compounds easier. In Fig. 1A the group-specific fragments are more intense. However, these effects have not been investigated in detail and more elaborate considerations of these effects will be given elsewhere[18].

The influence of some of the parameters was studied systematically. The results of these investigations are discussed here, demonstrating some of the striking features observed.

Changing the repeller potential from 0 to 100 V does not change the fragmentation pattern significantly; only the absolute intensities of the peaks are somewhat influenced. Optimization can give an increase in intensity of a factor of 2–3

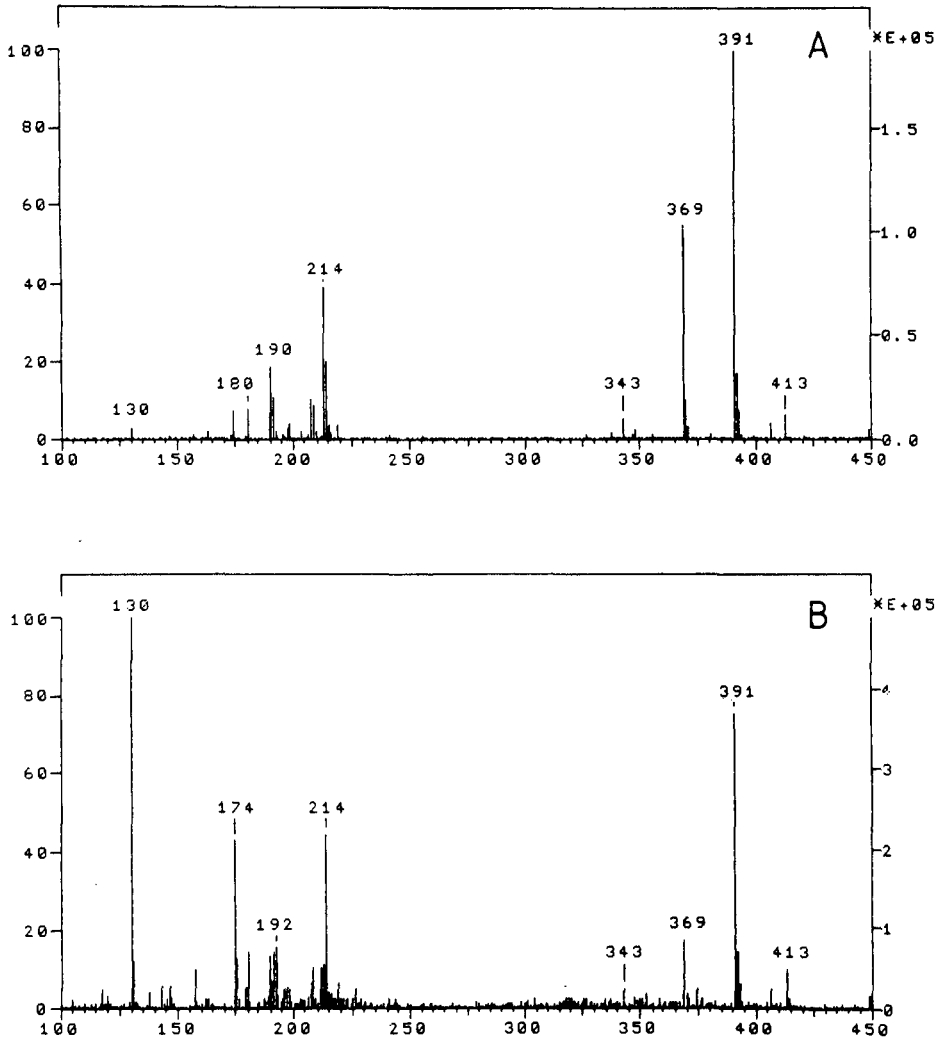


Fig. 2. TSP mass spectra of desulphoglucobrassicin, obtained at different vaporizer temperatures. (A) 100°C and (B) 130°C. Repeller potential, 50 V; modifier, methanol.

at most. Above about 100 V the peak intensity decreases dramatically. The optimum repeller potential is dependent on the contamination of the repeller, as this changes the effective potential. With a clean repeller electrode the optimum intensities are obtained at lower potentials than with a contaminated repeller electrode. The influence of the flow-rate and the vaporizer temperature was also investigated. When the vaporizer temperature is increased at a constant flow-rate, more fragmentation will be induced. This is especially important when the compounds studied are thermolabile, such as desulphoglucosinolates. For instance, at a flow-rate of 2.0 ml/min and a vaporizer temperature of 100°C the sodium cationized molecule $[M + Na]^+$ of glucobrassicin (*m/z* 391) is the base peak and hardly any fragmentation is observed. On increasing the temperature, fragmentation also increases, *e.g.*, at a vaporizer temperature of 130°C the fragment at *m/z* 130, $[R]^+$, has become the base peak (see Fig. 2), probably resulting from thermal degradation.

After optimization of the various parameters, TSP LC–MS was applied to a sprout extract. Because all of the desulphoglucosinolates studied showed a relatively intense peak at *m/z* 214, irrespective of the ionization conditions, screening for glucosinolates was performed with a mass chromatogram of this mass-to-charge ratio. In Fig. 3 a mass chromatogram (*m/z* 214) of sprout extract is shown, in which at least six desulphoglucosinolates can be observed. The mass spectra obtained confirm the structures of progoitrin, sinigrin, gluconapin and glucobrassicin, which are expected to be present by virtue of the chromatographic retention data. The other two peaks might be glucoiberin and neo-glucobrassicin, but the evidence is not very convincing, because the characteristic peaks, such as $[M + H]^+$ or $[RNCO + NH_4]^+$ are very small or even absent. No other desulphoglucosinolates than those expected from the retention data of standard compounds are observed. Screening for desulphoglucosinolates using this approach is possible, but the identification of observed desulphoglucosinolates in a mixture is not always straightforward.

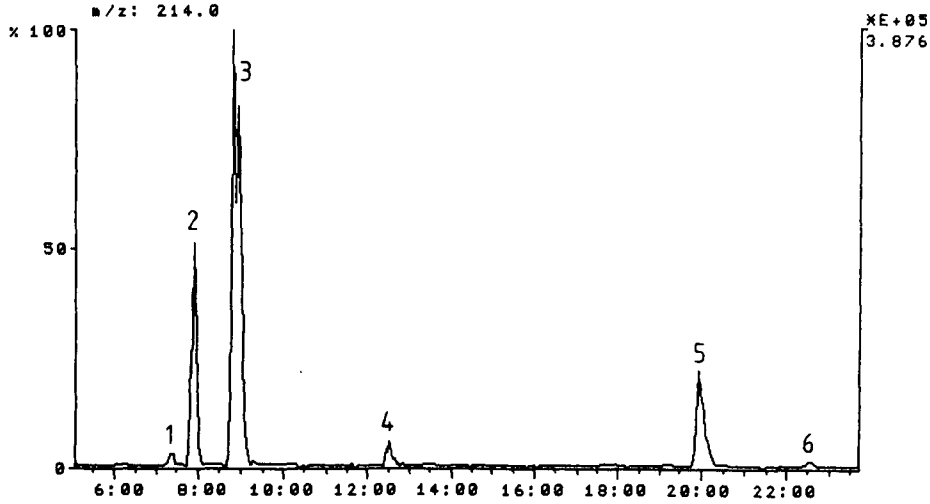


Fig. 3. Mass chromatogram (*m/z* 214) of a sprout extract. Modifier, acetonitrile; repeller potential, 50 V; vaporizer temperature, 100°C. Peaks: 1 = glucoiberin (?); 2 = progoitrin; 3 = sinigrin; 4 = gluconapin; 5 = glucobrassicin; 6 = neo-glucobrassicin (?).

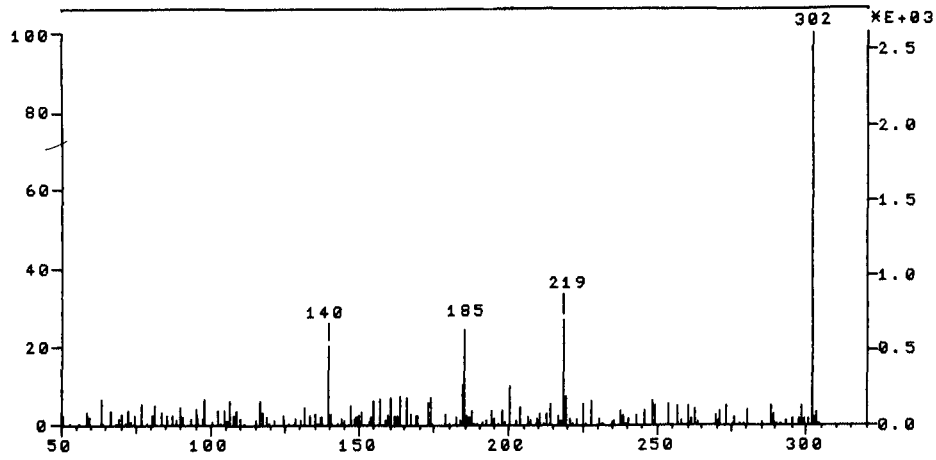


Fig. 4. Daughter spectrum of the sodium cationized molecule of desulphosinigrin (*m/z* 302). Collision gas, on; collision energy, 30 V.

Because of the increased selectivity, MS-MS in combination with HPLC was also investigated for the analysis of desulphoglucosinolates. Daughter spectra were obtained from the protonated and sodium cationized molecules of various desulphoglucosinolate standards. Fig. 4 shows the daughter spectrum of the sodium cationized molecule of sinigrin (*m/z* 302). Three fragments are formed at *m/z* 140, 185 and 219, which correspond to $[M + Na - C_6H_{10}O_5]^+$, $[C_6H_{10}O_5 + Na]^+$ and $[M + Na - RNCO]^+$, respectively. In the daughter spectrum of the protonated molecule of sinigrin (*m/z* 280), one intense fragment at *m/z* 118 is observed (see Fig. 5), which

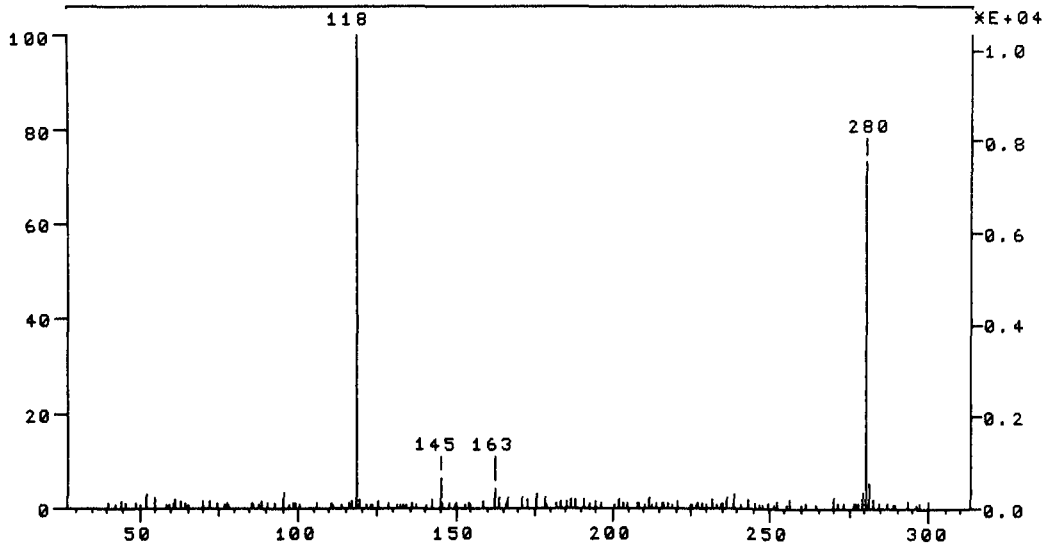


Fig. 5. Daughter spectrum of the protonated molecule of desulphosinigrin (*m/z* 280). Collision gas, off; collision energy, 20 V.

corresponds to a loss of the sugar ring ($C_6H_{10}O_5$) (162 a.m.u.). The daughter spectra of the sodium cationized molecule and the protonated molecule both show a loss of the sugar ring (162 a.m.u.) resulting in fragment ions at *m/z* 140 and 118. All the desulphoglucosinolates studied show a similar fragmentation of the protonated molecule under these conditions, *i.e.*, a loss of 162 a.m.u. A common fragmentation in the collision-induced dissociation spectra of this class of compounds indicates the possibility of using the neutral loss scan mode in tandem mass spectrometry. Therefore, LC–MS–MS in the neutral loss mode, *i.e.*, Q1 and Q3 both scanning with a mass difference of 162 a.m.u., was performed on various desulphoglucosinolate standards and the sprout extract. Fig. 6 shows the mass chromatograms based on the neutral loss experiments on the protonated molecules of various desulphogluco-

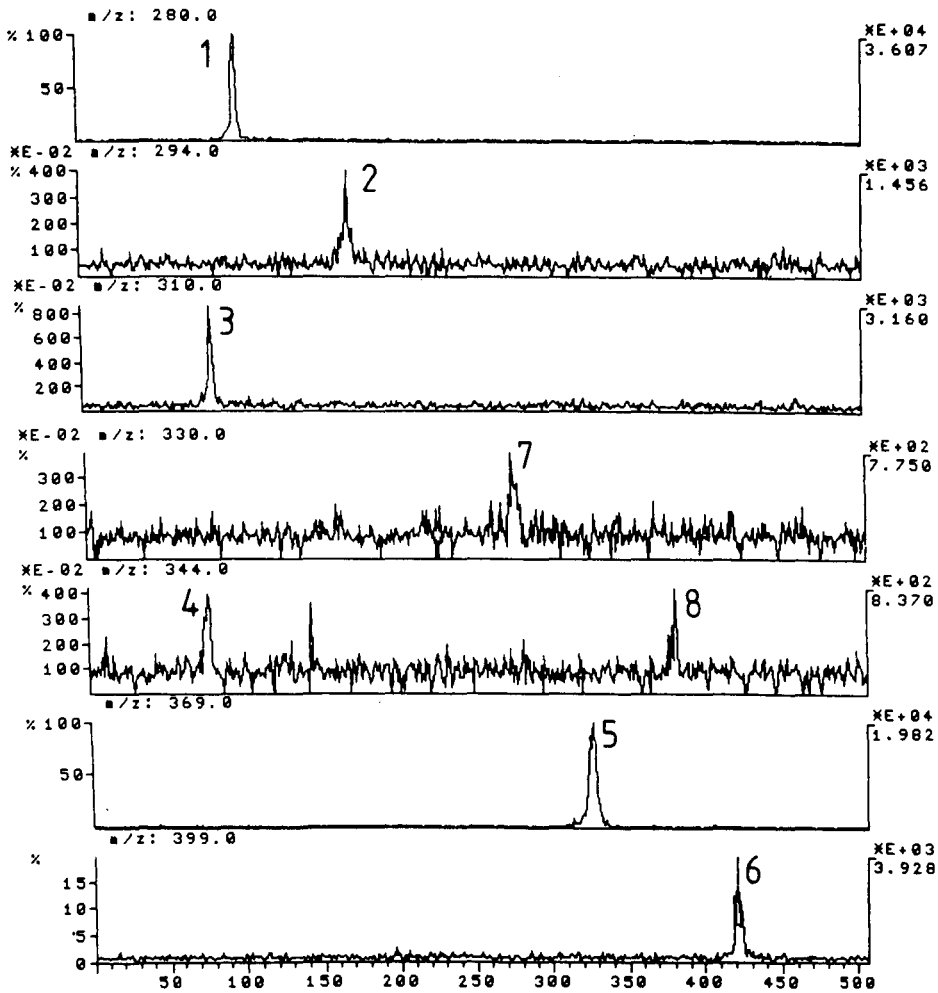


Fig. 6. Mass chromatograms of the protonated molecules of desulphoglucosinolates in a sprout extract obtained in the neutral loss (162 a.m.u.) scanning mode. Peaks: 1 = sinigrin; 2 = gluconapin; 3 = progoitrin; 4 = glucoiberin; 5 = glucobrassicin; 6 = neo-glucobrassicin; 7 = glucotropaeolin; 8 = gluconasturtiin. Collision gas, on; collision energy, 30 V.

sinolates observed in the sprout extract. Although the neutral loss scan mode is less dependent on the TSP ionization conditions, the presence of the protonated molecule is obligatory. The instrument used in these experiments displays the mass of the parent ion in the recorded mass spectra or mass chromatograms and not the mass of the detected daughter ion. Six desulphoglucosinolates can be identified at once, *viz.*, glucoiberin, progoitrin, sinigrin, gluconapin, glucobrassicin and neo-glucobrassicin, the presence of which was also suggested using HPLC with UV detection. Progoitrin, sinigrin, gluconapin and glucobrassicin were also identified in the MS mode, but for the presence of glucoiberin and neo-glucobrassicin there was only some slight evidence. A more detailed search of the MS–MS data also gives some evidence for the presence of glucotropaeolin and gluconasturtiin ($[M + H]^+$, m/z 330 and 344, respectively). For the presence of glucocapparin (m/z 254) (not shown in Fig. 6) there is only a slight indication. Our HPLC analysis with UV detection failed to detect these glucosinolates, probably owing to their low concentrations. The presence of gluconasturtiin in Brussels sprouts has been reported[3].

The enzymatic desulphation procedure appears to be a selective sample pretreatment, resulting in clean samples, containing few components that will rapidly contaminate the ion source. Therefore, the direct analysis of sprout extracts by means of TSP MS–MS, operating in the bypass or flow-injection mode, can be considered. The mass spectrum obtained by injecting the sprout extract directly without chromatographic separation and analysing in the neutral loss scan mode as earlier is shown in Fig. 7. Various desulphoglucosinolates can be identified rapidly in this way, *e.g.*, sinigrin from the peak at m/z 280, gluconapin from m/z 294, progoitrin from m/z 310 and glucobrassicin from m/z 369. However, because of the large differences in the concentrations of the various desulphoglucosinolates in the sprout extract, and because the sodium cationized molecules show a similar neutral loss of 162 a.m.u., the identification of minor components is not easy. Further, isomeric compounds cannot be differentiated by the flow-injection procedure. The selected reaction monitoring

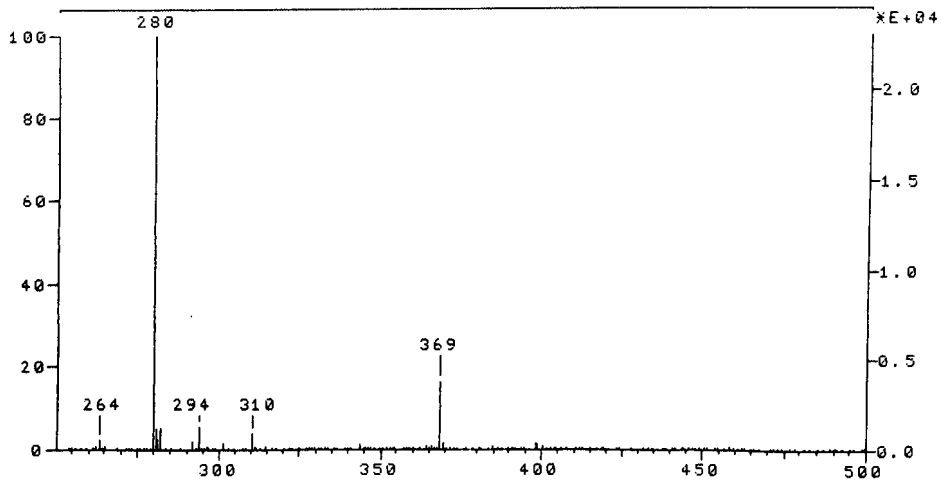


Fig. 7. MS–MS trace of a sprout extract obtained by flow injection in the neutral loss (162 a.m.u.) scanning mode.

mode (SRM) can be used to increase the selectivity further for certain applications. The choice between very rapid and simple direct analysis in with the flow-injection mode, taking only a few minutes, and the chromatographic analysis, taking about 1 h, will depend on the specific problem involved.

CONCLUSIONS

The combination of reversed-phase HPLC and either MS or MS–MS has been demonstrated to be a very useful method for the detection and identification of desulphoglucosinolates in sprout extracts.

In the MS mode sprout extracts can be easily screened for the presence of desulphoglucosinolates by the use of *m/z* 214 mass chromatograms. Identification of unknown compounds is not always straightforward, especially as some of the experimental parameters can exert a large influence on the TSP mass spectra. In the MS–MS mode desulphoglucosinolates can be identified owing to the monitoring of a highly selective reaction in the neutral loss scan mode, *i.e.*, the loss of the sugar ring (162 a.m.u.). Depending on the purpose, the analysis of desulphoglucosinolates can be performed by either chromatographic separation or direct analysis in the flow-injection mode, with the tandem mass spectrometer used in the neutral loss (162 a.m.u.) mode, either scanning or selective reaction monitoring.

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DETERMINATION OF N-NITROSODIETHANOLAMINE IN COSMETICS

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY AS ALTERNATIVE METHODS TO CHEMILUMINESCENCE DETECTION

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SUMMARY

For the determination of N-nitrosodiethanolamine (NDELA) in cosmetic products chemiluminescence detection has been widely adopted, but two alternative techniques are now considered. A high-performance liquid chromatographic method using UV detection can be used for the routine determination of underivatized NDELA. A gas chromatographic–mass spectrometric method was also studied. Different derivatives of NDELA and several ionization methods were examined to match the selectivity and sensitivity given by the chemiluminescence detector.

INTRODUCTION

The possible presence of N-nitrosamines and their formation in cosmetic products have created a continuing need for the detection and positive identification of low levels, and numerous methods have been developed[1–6]. The thermal energy analyser, a chemiluminescence detector, is today the most popular detector for nitrosamine analysis. It provides excellent selectivity, combined with detection limits as low as 1–5 ng for most of the compounds. However, the operation of this detector is cumbersome and requires a skilled analyst. As thermal energy analysers are expensive instruments and dedicated to the analysis of nitrosamines, many analysts have attempted to explore other, simpler methods for the detection of these compounds in cosmetic samples[4–6]. These simplified methods can be particularly useful in quality control laboratories in the industry, where a deep knowledge of the sample to be analysed is available.

Based on an extraction and clean-up procedure developed by Sommer and Eisenbrand[7], we have explored the use of a direct high-performance liquid chromatographic (HPLC) method with UV detection, which proved suitable for detecting NDELA at low levels (5 ppb) in known products. As a further alternative to chemiluminescence detection, gas chromatography–mass spectrometry (GC–MS) of NDELA derivatives was examined. This method is highly specific and gives structural information for the positive identification of NDELA.

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EXPERIMENTAL

HPLC system

An M-6000 pump (Millipore Waters, Milford, MA, U.S.A.) was used to force the mobile phase (distilled water) through a 250 × 4.6 mm I.D. stainless-steel column packed with LiChrosorb RP-18, 5 μm (Merck, Darmstadt, F.R.G.) at room temperature. An M-440 UV detector (Millipore Waters) was used to monitor the eluate at 254 nm. To obtain low detection limits, the whole system must be operated under its optimum performance conditions. The water must be degassed carefully in an ultrasonic bath using argon. New columns must be purged with methanol before use to ensure complete wetting of the support surface.

GC system

A Fractovap 2150 gas chromatograph (Carlo Erba, Milan, Italy) was modified for on-column injection, equipped with a glass capillary column (laboratory-made, SE-30, film thickness 0.13 μm, 28 m × 0.3 mm I.D.) and operated at a carrier gas (helium) inlet pressure of 0.8 atm. Injections were made on-column at 100°C. The oven temperature was kept at 100°C for 3 min, then increased at 7°C/min to 240°C.

Mass spectrometer

A Finnigan-MAT (Bremen, F.R.G.) Model 212 mass spectrometer equipped with a combined electron impact–chemical ionization (EI–CI) source was coupled to the GC system by means of an open split interface. The instrument was connected to a Finnigan-Incos data system which controlled all accelerating voltage selected ion monitoring (SIM) scans. For accelerating negative ions a Bertan Model 205A-03R high-voltage power supply was used. The ion source temperture was 250 and 180°C in the EI and CI mode, respectively. Methane (CH4 45) and ammonia (N36) (Carbagas, Rümlang, Switzerland) were used as reactant gases in the CI mode.

Sample preparation

Cosmetic products. The method described by Sommer and Eisenbrand[7] was used for a wide range of cosmetic products. In some instances it was difficult to break the emulsion and the procedure was partly modified. However, clean extracts suitable for HPLC analysis or derivatization followed by GC–MS were obtained in most instances.

A 2-g amount of cosmetic product is mixed with 9 ml of distilled water and 0.5 g of ammonium sulphamate (Fluka, Buchs, Switzerland) is added. To break up the emulsion the mixture is saturated with sodium chloride (Fluka) and 1.5 ml of chloroform (Merck) are added. The mixture is placed on a glass column (20 × 3 cm I.D.) packed with 12 g of Extrelut (Merck). The mixture is allowed to penetrate into the adsorbent for 20 min, then the column is rinsed with 100 ml of cyclohexane–dichloromethane (1:1, v/v) and subsequently connected to a stream of nitrogen to force out all solvents. The NDELA fraction is then eluted with 150 ml of *n*-butanol (Merck). The eluate is evaporated to dryness.

A second glass column (20 × 3 cm I.D.) packed with sodium sulphate–silica gel is prepared as follows: 6 g of sodium sulphate are suspended in methanol–acetone (1:1) and placed at the bottom of the column, then 10 g of silica gel 40 (0.063–0.200 mm; Merck) in methanol–acetone (1:1) are carefully placed on top of the sodium sulphate

layer. The column is washed with *ca.* 20 ml of chloroform–acetone (5:1). The dry residue is dissolved in 20 ml of chloroform–acetone (5:1) and placed on top of the column, which is washed with 60 ml of chloroform–acetone (5:1) and then eluted with 50 ml of acetone. The final eluate is evaporated to dryness and the residue is used for direct analysis by HPLC or derivatized for GC–MS identification.

Alkanolamines. For the determination of NDELA in alkanolamines, a strong cation exchanger was used to bind all the amine according to the procedure proposed by Sommer *et al.*[8].

A 1-g amount of amine (*e.g.*, diethanolamine or triethanolamine) is dissolved in 10 ml of water–methanol (1:1). A 0.2% solution of methyl red (Fluka) in water–methanol (1:1) as indicator is added until the solution is yellow. A cation exchanger in the H^+ form (Amberlite IR-120, 16–45 mesh; Fluka) is added until the colour of the solution changes to red. More cation exchanger is added to adsorb the indicator and to produce an almost colourless solution (20–30 ml are typically used). This suspension is filled in a glass column (20 × 3 cm I.D.) and eluted with 100 ml water–methanol (1:1).

The eluate is evaporated to dryness and the residue is dissolved in 20 ml of chloroform–acetone (5:1) and placed on a glass column (20 × 3 cm I.D.) packed with sodium sulphate–silica gel (this column is prepared in the same way as described above for the determination of NDELA in cosmetic products). The silica gel column is washed with an additional 60 ml of chloroform–acetone (5:1) and finally eluted with 50 ml of acetone. The acetone eluate is evaporated to dryness and the residue can be used for direct separation on a HPLC system or derivatized for GC–MS.

Derivatization. The dry residue is dissolved in 200 μl of MSTFA reagent (N-methyl-N-trimethylsilyltrifluoracetamide; Fluka) and kept at room temperature for 45 min. The reaction mixture is then diluted to 0.5 ml with isooctane (Fluka) and aliquots are analysed by GC–MS for the NDELA bistrimethylsilyl (bis-TMS) ether (see formula II below).

Formation of the NDELA bis-*tert.*-butyldimethylsilyl (bis-tBuDMS) ether (see formula III below) is accomplished by reaction of the dry residue with *tert.*-butyldimethylchlorsilane imidazole reagent (Alltech/Applied Science, Rockwood, Canada) (0.5 ml) at 60°C for 30 min. The reaction mixture is diluted with 1 ml of water and extracted three times with hexane. The hexane extracts are subsequently evaporated to dryness and the residue is dissolved in 0.5 ml of isooctane.

RESULTS AND DISCUSSION

Direct determination by HPLC using UV detection

UV detection of NDELA after HPLC separation is an attractive alternative to chemiluminescence detection because it is simple and allows some of the present limitations of the thermal energy analyser detector to be overcome[9]. Since the early paper of Mitchell and Rhan[10], many analysts have successfully used direct UV detection. Wigfield and Lanouette[11] compared the sensitivity of UV and thermal energy analysis detection and demonstrated clearly that the signal-to-noise ratio in these two techniques is about the same for pure NDELA. In a complicated matrix, however, the determination of small amounts of NDELA is better accomplished by the thermal energy analyser detector owing to its high specificity. Therefore, the better the clean-up, the greater is the possibility of determining NDELA directly by HPLC with

UV detection. As the extraction and clean-up procedure described under Experimental leads to a sample matrix that is sufficiently free from interferences, NDELA can be determined at low ppb levels.

The UV absorption spectrum of NDELA is shown in Fig. 1. The maximum absorbance of this compound is at 234 nm. Using 254 nm in a single-wavelength UV detector, only about 50% of the signal is available. However, at 254 nm fewer interfering compounds absorb and therefore the signal-to-noise ratio may be even better. The transparency of the mobile phase is also higher at longer wavelengths and therefore more energy is available, which leads to a better signal-to-noise ratio. In the examples shown in Fig. 2, 254 nm is used as the detection wavelength. Fig. 2a shows 30 ppb[a] of NDELA in water, worked-up as a sample. The recovery was above 90%. A blank water sample (Fig. 2b) shows no interference in the eluting range of NDELA. Fig. 2c and d show chromatograms of an emulsion without and with 50 ppb of NDELA, respectively. The recovery was in the range 65–80% for this type of product. For shower shampoos, as shown in Fig. 2e and f, the recovery was better than 90%. The shampoo in Fig. 2e was spiked with 50 ppb of NDELA. These chromatograms show the efficiency of the clean-up technique for the HPLC analysis of real samples.

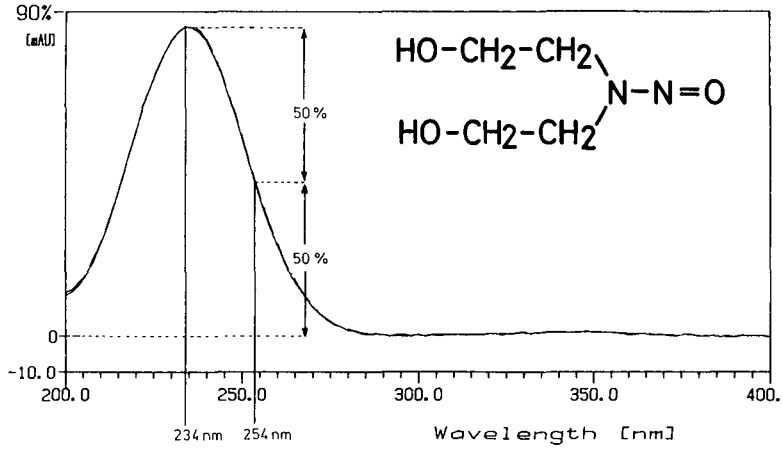


Fig. 1. UV absorption spectrum of N-nitrosodiethanolamine (mol.wt. 134).

The HPLC separation is achieved on a reversed-phase column. Only LiChrosorb RP-18 gives a reasonable retention for NDELA with water as the mobile phase. HPLC with organic mobile phases on silica gel or polar bonded phases is also possible, but such systems are not as easily equilibrated and tend to give lower signal-to-noise ratios.

The NDELA peak is identified by its retention time. Its concentration in the ppb range is too low for identification by a modern photodiode array detector. However, the retention time may be influenced by many factors and the possibility of co-elution with other compounds is high. To overcome this drawback, we used the extreme instability of NDELA to UV light to confirm its identity. The injection solution is

[a] Throughout this article, the American billion (10^9) is meant.

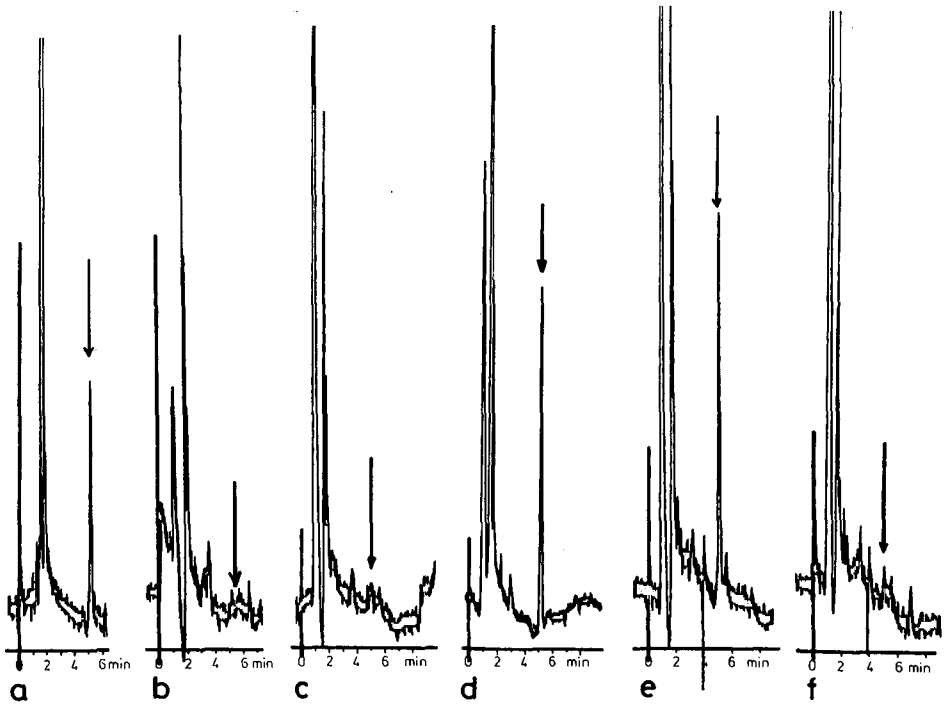
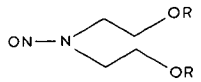


Fig. 2. Examples of HPLC with UV detection at 254 nm: (a) 30 ppb of NDELA in water; (b) blank water; (c) blank emulsion; (d) emulsion containing 50 ppb of NDELA; (e) shampoo containing 50 ppb of NDELA; (f) blank shampoo. Chromatographic conditions: column, LiChrosorb RP-18, 5 μm (250 × 3 mm I.D.); room temperature; mobile phase, water at a flow-rate of 1.0 ml/min.

placed in a quartz cuvette and irradiated for 5 min with a low-power mercury lamp. The disappearance of the peak corresponding to NDELA is taken as good evidence for its identity. This additional test can be used to minimize the possibility of analytical artifacts.

GC–MS of NDELA derivatives

Owing to the chromatographic behaviour and lack of thermal stability of the compound, derivatization of NDELA is essential for GC–MS determinations. NDELA bis-TMS ether (formula II), the most simple silylated derivative of NDELA, is easily formed by reaction with MSHFBA, MSTFA[7] or BSA[11]. For mass spectrometric reasons, the use of alternative silylated derivatives of NDELA such as NDELA bis-tBuDMS ether[12] (formula III) may be more useful.



I R = H(NDELA)
II R = $Si(CH_3)_3$
III R = $Si(CH_3)_2$-*tert.*-C_4H_9

Full scan mass spectra of compounds II and III were recorded under different ionization conditions. The resulting spectra are displayed in Figs. 3 and 4. The EI mass spectra of the two compounds (Figs. 3a and 4a) compare well with the spectra given by

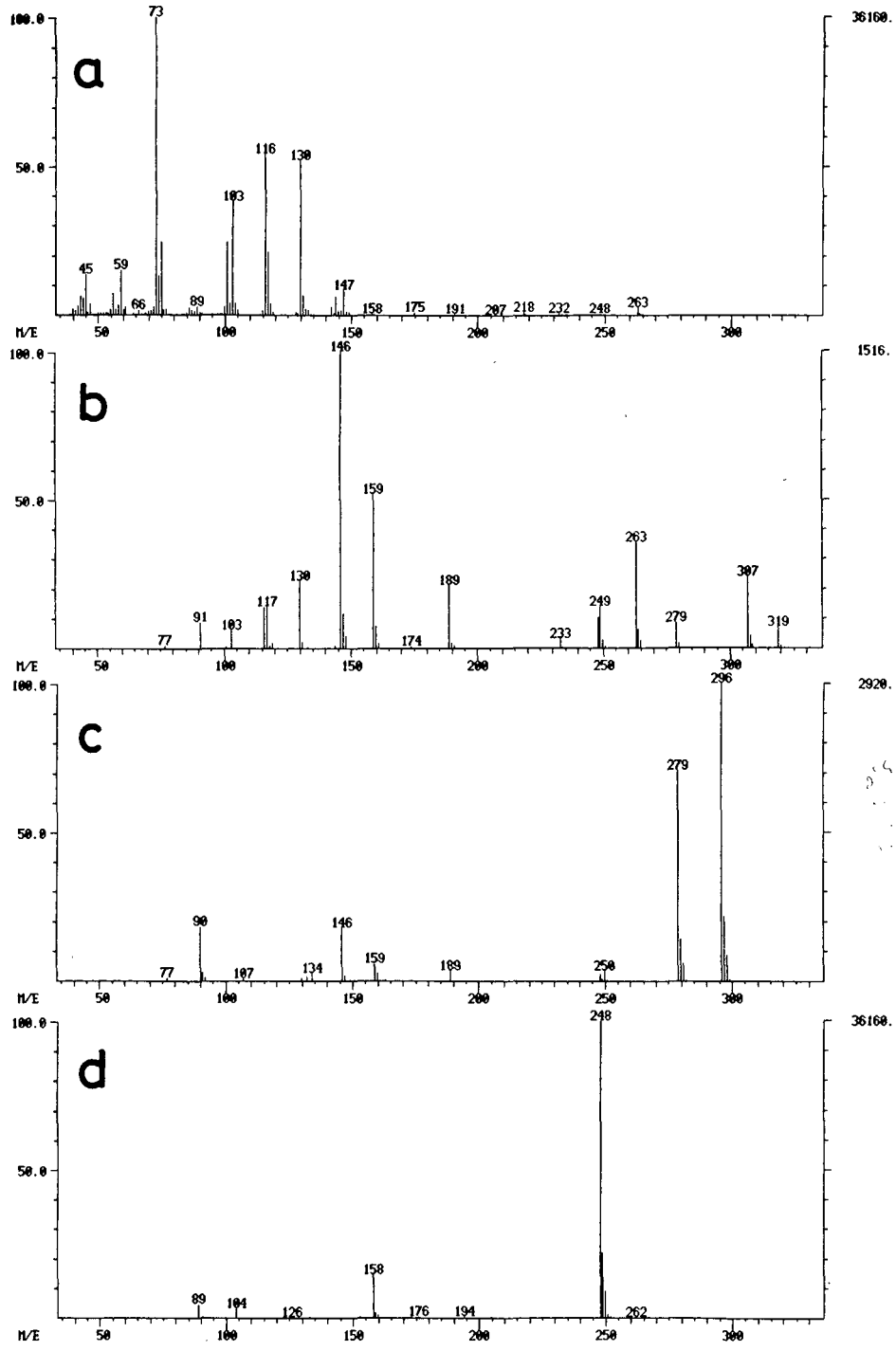


Fig. 3. Mass spectra of II (N-nitrosodiethanolamine bistrimethylsilyl ether, MW 278) using (a) EI, positive ion CI with (b) methane or (c) ammonia and (d) negative ion CI with methane.

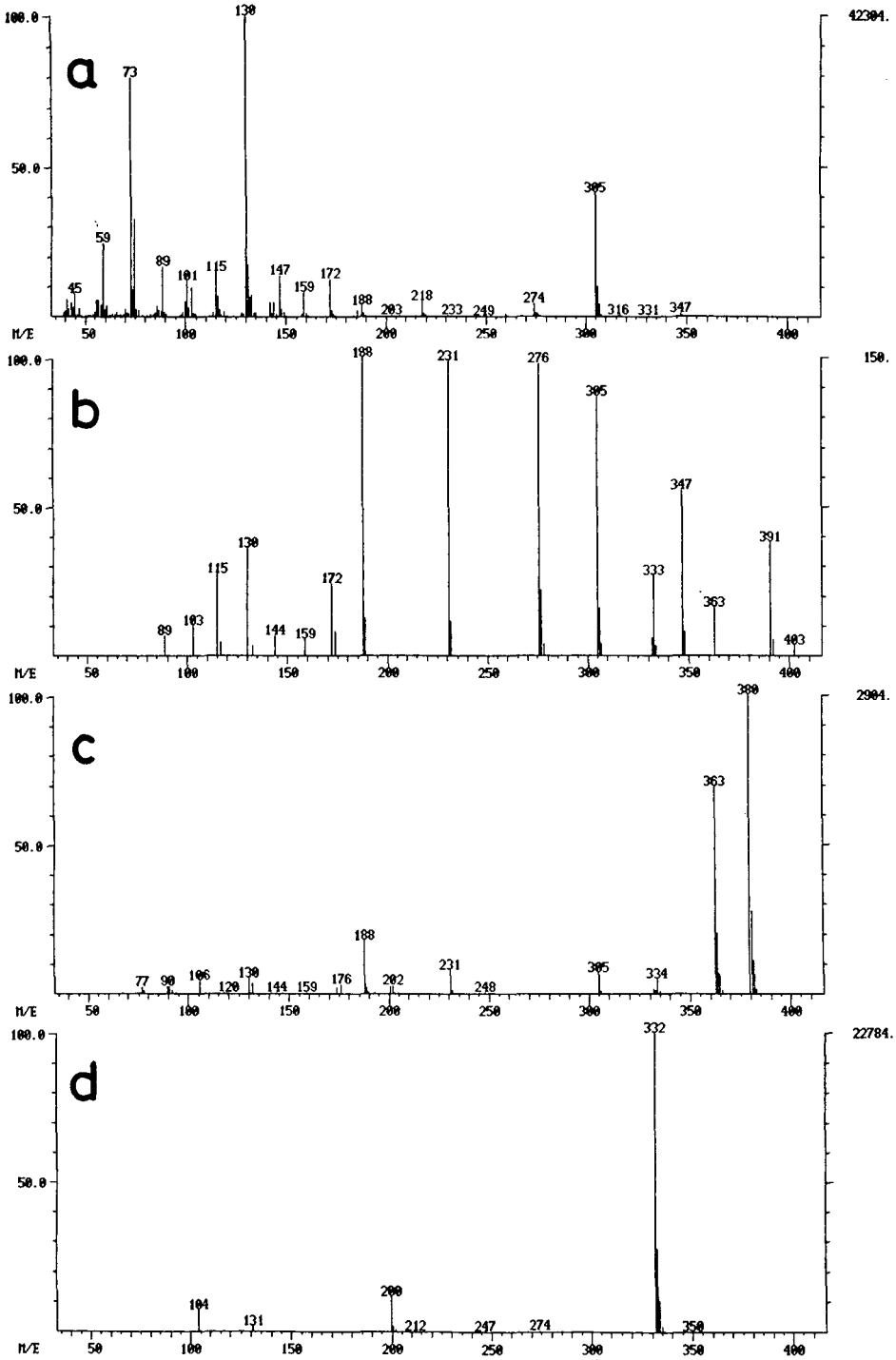


Fig. 4. Mass spectra of III (N-nitrosodiethanolamine bis-*tert.*-butyldimethylsilyl ether, MW 362) using (a) EI, positive ion CI with (b) methane or (c) ammonia and (d) negative ion CI with methane.

Issenberg *et al.*[12]. Although the two derivatives show a similar total ion yield, the distributions of peaks in their EI mass spectra differ considerably. Fig. 3a for compound II shows only a very low abundance at the structurally most characteristic peak of *m/z* 263 (loss of CH_3 from the molecular ion), but includes many strong peaks indicating merely side-chain fragments (*e.g.*, *m/z* 73, C_3H_9Si; *m/z* 103, $C_4H_{11}OSi$; *m/z* 116, $C_5H_{12}OSi$). Fig. 4a for compound III provides a signal of similar structural significance at *m/z* 305 (loss of C_4H_9 from the molecular ion) which, however, is of much higher abundance (*ca.* 45% of the base peak) and therefore much better suited for use in SIM measurements.

Methane positive ion CI mass spectra of the two compounds (Figs. 3b and 4b) are of little interest for the purpose of quantitation or for providing structural information, as the total ion yield is very low and concurrently a wide variety of fragment peaks with little significance are observed, the pseudo-molecular ions (*m/z* 279 and 363 respectively) being of almost no importance. A totally different situation is encountered in the methane negative ion CI mass spectra (Figs. 3d and 4d), where high-intensity peaks are measured for both compounds. The spectra display a base peak that corresponds to a loss of NO from the molecular ion and presumably is formed through a dissociative electron-capture process[13]. Thus the peaks at *m/z* 248 and 332 are excellent possibilities for use in SIM quantifications. For compound II these data are in good agreement with the spectra given by Wigfield and Lanouette[11].

The ammonia positive ion CI mass spectra (Figs. 3c and 4c) were measured in order to complete the information available about the positive ion CI of those compounds. In contrast to methane CI, qualitatively good spectra with high-abundance pseudo-molecular ion peaks of $M+H$ and $M+NH_4$ were observed. The overall sensitivity in this ionization mode, however, turned out to be low in preliminary experiments.

SIM trace determinations of NDELA derivatives

EI SIM measurements on the NDELA bis-TMS-derivative II were tried but turned out to be very unsatisfactory, as can easily be understood from examination of Fig. 3a. The SIM traces on all the mass peaks at *m/z* 103, 116, 130 or 263 included a large number of interfering GC peaks and also showed an elevated baseline due to ion noise, thus leading to an unfavourable signal-to-noise ratio. This situation was encountered with both standard injections and injections of cosmetic emulsion samples. Even increasing the mass spectrometric resolution to $m/\Delta m = 4000$ only slightly improved the results. Switching the ion source to methane negative ion CI provided the selectivity and sensitivity necessary for the analysis of cosmetic emulsions (Fig. 5): injections of an emulsion sample containing 50 ppb of NDELA (Fig. 5b) and of a standard sample with an identical amount of NDELA (Fig. 5a) resulted in very similar SIM mass chromatograms, practically free from interfering peaks, thus indicating the excellent selectivity of the method[14]. Comparison of the analysis of a sample with an unknown NDELA content (*ca.* 7 ppb, Fig. 5c) with the result for a blank injection (Fig. 5d) indicates a good signal-to-noise ratio.

Results of EI SIM analyses of the NDELA bis-tBuDMS derivative III are shown in Fig. 6. The mass chromatograms are not free from interfering peaks from the derivatization step (Fig. 6a) or the emulsion matrix (Fig. 6b–d), but the selectivity obtained by scanning the *m/z* 305 mass peak has drastically increased as compared

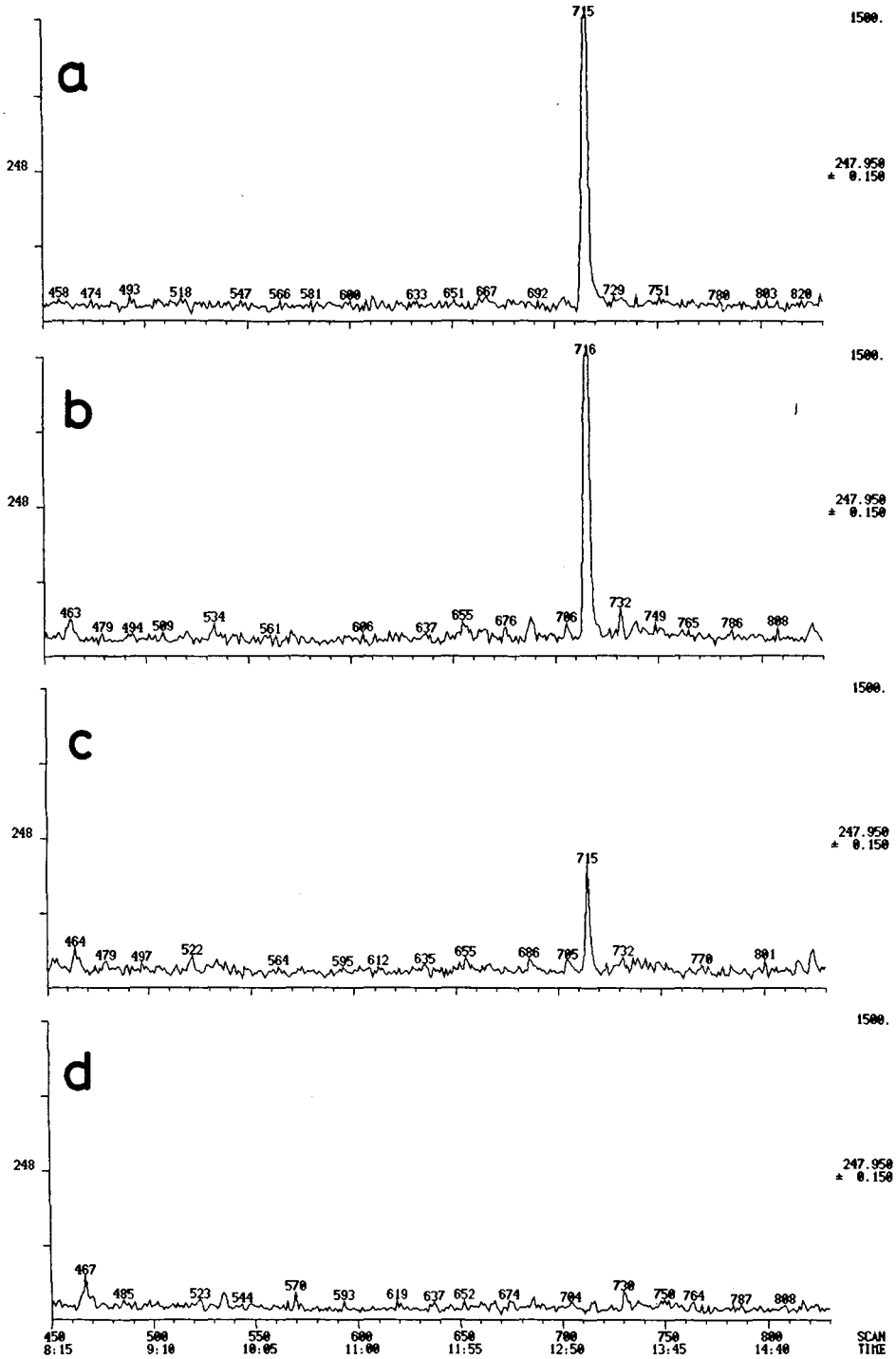


Fig. 5. Negative ion CI (methane) SIM mass chromatograms for m/z 248 ($m/\Delta m$ = 1000). Samples (derivatized with MSTFA): (a) NDELA standard (420 pg of derivative, corresponding to 50 ppb in an injection aliquot); (b) emulsion, after clean-up, containing 50 ppb of NDELA; (c) emulsion, after clean-up, with unknown amount of NDELA; (d) blank (solvent and derivatization reagent). Uniform attenuation in all instances.

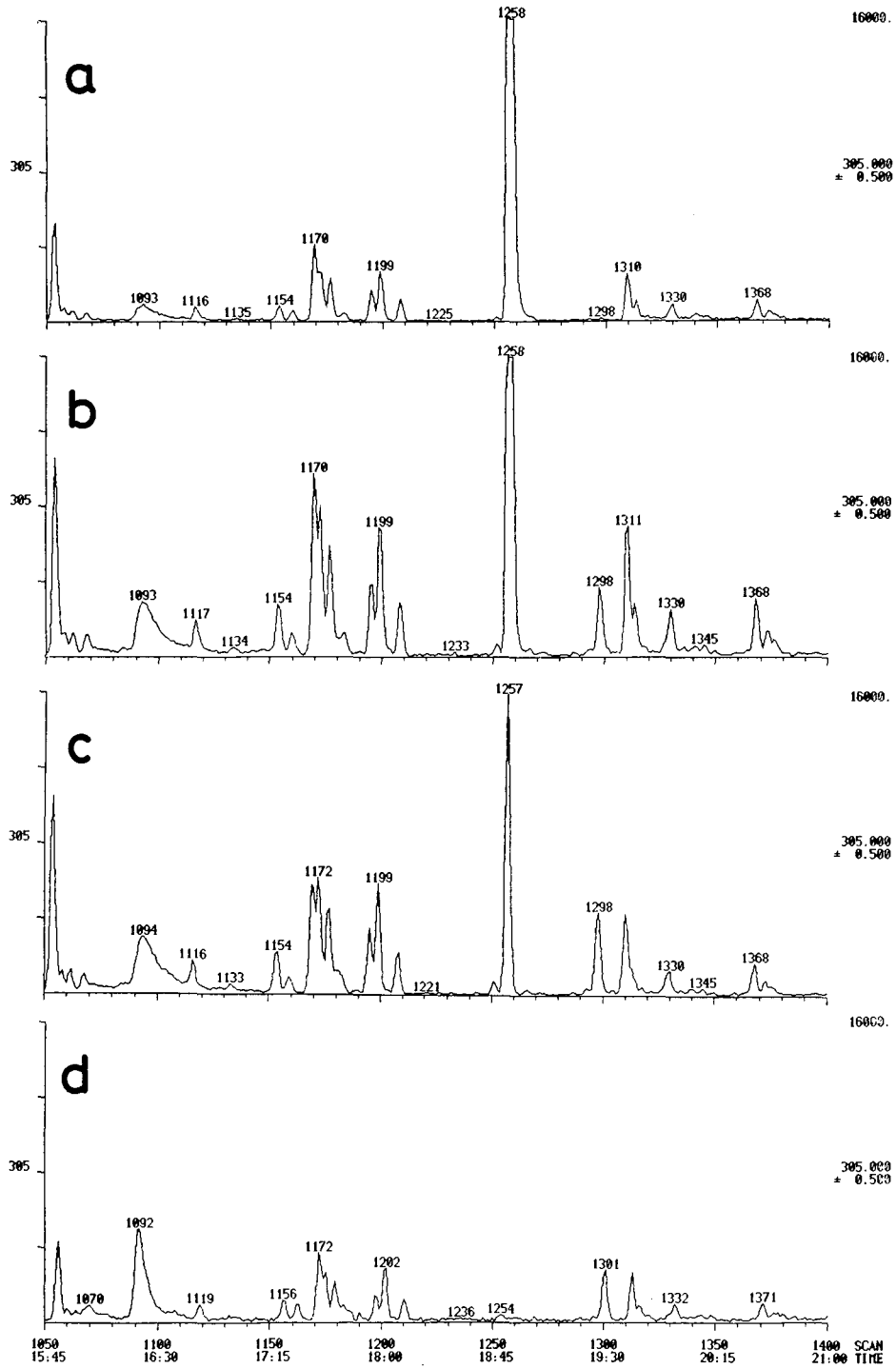


Fig. 6. EI SIM mass chromatograms for m/z 305 ($m/\Delta m$ = 1000). Samples (derivatized with t-BuDMCS–imidazole): (a) NDELA standard (540 pg of derivative, corresponding to 50 ppb in an injection aliquot); (b) emulsion, after clean-up, containing 50 ppb of NDELA; (c) emulsion, after clean-up, with unknown amount of NDELA; (d) sample (c) after irradiation for 30 min with UV light. Uniform attenuation in all instances.

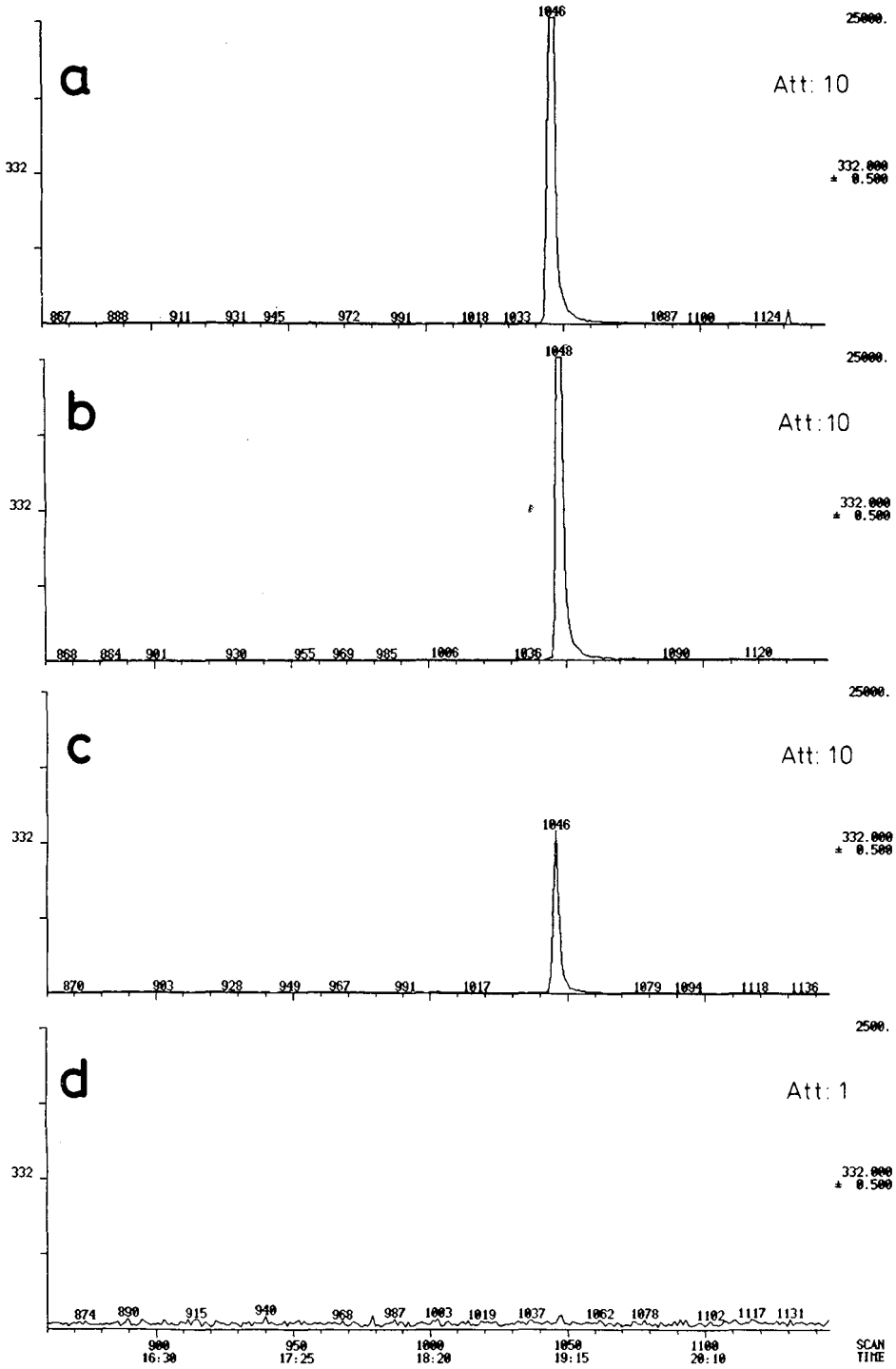


Fig. 7. Negative ion CI (methane) SIM mass chromatograms for m/z 332 ($m/\Delta m$ = 1000). Samples (derivatized with t-Bu DMCS–imidazole): (a) NDELA standard (540 pg of derivative, corresponding to 50 ppb in an injection aliquot); (b) emulsion, after clean-up, containing 50 ppb of NDELA; (c) emulsion, after clean-up, with unknown amount of NDELA; (d) sample (c) after irradiation for 30 min with UV light. Attenuation as indicated.

with EI SIM analyses of derivative II, and it facilitates the determination of an estimated 15 ppb of NDELA with a very good signal-to-noise ratio (Fig. 6c). Fig. 6d was obtained after UV irradiation of an injection aliquot of the sample from Fig. 6c, thus giving a measure of the noise level in the mass chromatograms and further proving the identity of the GC peak with a retention time of 18.75 min.

For the derivative III, a similar sensitivity in methane negative ion CI to that for derivative II could be expected from comparison of the full scan spectra (Figs. 3d and 4d). However, a better signal-to-noise ratio due to the mass shift of 84 units to a mass peak with less background ion noise at m/z 332 could also be anticipated. This indeed occurs, as illustrated very clearly by the SIM mass chromatograms of standard and cosmetic emulsion samples in Fig. 7. Again there are no interfering peaks present, and the chromatogram obtained from the UV-irradiated, and therefore NDELA-free, sample (attenuation factor ten times lower) demonstrates an excellent signal-to-noise ratio. For the sample with an unknown NDELA content (*ca.* 15 ppb, Fig. 7c), this ratio was calculated to be about 200:1.

CONCLUSION

The determination of NDELA still receives attention in spite of the many methods available today. Two alternative means of determining NDELA at the ppb level have been presented here. The mass spectrometer, operated in the negative ionization mode, has been shown to be more sensitive than the thermal energy analyser detector. UV detection after HPLC separation proved to be an easy method for routine determinations of NDELA in known products. The success of these methods was made possible by the use of an efficient clean-up procedure[7,8].

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IDENTIFICATION OF ADP-RIBOSYLATED HISTONES BY THE COMBINED USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROPHORESIS

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SUMMARY

Reversed-phase high-performance liquid chromatography (HPLC) was employed for analysing mono- and oligo(ADP-ribosyl)ated histones. Under the chromatographic conditions described, the ADP-ribosylated histones showed similar retention times to the unmodified histones, although the molecular weight and the charge of the proteins are significantly altered by their modification. The simultaneous elution of unmodified and labelled modified histones was detected by two types of gel electrophoresis and by autoradiography. In addition, the HPLC fractions did not display overlapping ladders of the multiply modified histones, as is commonly seen in one-dimensional electrophoretic analyses of unfractionated material. Hence individual bands could be unambiguously assigned. After *in vitro* labelling of isolated rat liver nuclei, the following ADP-ribosylated and unmodified histones were identified by HPLC and gel electrophoresis: histone H1°, four histone H1 subfractions, histone H2A.1, histone H2A.2, oxidized histone H2A.2, histone H2A.X, histone H2A.Z, histone H2B, three histone H3 variants and histone H4.

INTRODUCTION

Under various biological conditions, histone molecules of eukaryotic cells are known to be modified in reactions catalysed by the DNA-dependent enzyme nuclear ADP-ribosyltransferase (E.C. 2.4.2.30). Although all main histone species have been reported to be capable of modification by covalent linkage to mono- or poly(ADP-

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ribose) residues, knowledge about the modification of histone variants is scarce (for reviews, see refs. 1 and 2).

ADP-ribosylated histones are usually identified by one-dimensional acrylamide gel electrophoreses[3–5] of the labelled compounds. As poly(ADP-ribose) chains of different lengths may be attached to the histone molecules, overlapping of histones and modified histones often excludes the identification of the individual components.

In this study, the behaviour of ADP-ribosylated histones during reversed-phase high-performance liquid chromatography (RP-HPLC)[6–8] was investigated. Experimental conditions were established that allowed the concomitant elution of the highly negatively charged ADP-ribosyl derivatives of individual histones or histone variants and their respective unmodified counterparts. Hence the individual ADP-ribosylated histone species can rapidly be detected and fractionated by RP-HPLC, prior to further analysis by gel electrophoresis. This permits the unambiguous assignment of the derivatives without the shortcomings outlined above. Moreover, the conditions were improved for the HPLC separation of some histone variants.

EXPERIMENTAL

HPLC-grade acetonitrile (grade S) and water were obtained from Rathburn Chemicals (Walkerburn, U.K.) and trifluoroacetic acid (TFA) was purchased from Sigma (Munich, F.R.G.).

Preparation of labelled ADP-ribosylated histones

Isolated rat liver nuclei[9] ($5 \cdot 10^8$ nuclei/ml) were incubated with 0.5 m*M* [adenylate-^{32}P]NAD (New England Nuclear) in the presence of 10 μg/ml DNAse I in medium A [100 m*M* Tris–HCl (pH 8.0), 10 m*M* magnesium chloride, 1 m*M* dithiothreitol (DTT), 1 m*M* phenylmethylsulphonyl fluoride (PMSF)] at 25°C for 30 min. The labelled nuclei were washed twice with medium A containing 5 m*M* unlabelled NAD at 4°C. The histones were then extracted with 0.2 *M* sulphuric acid at 4°C and precipitated by addition of 5 volumes of ethanol and overnight storage at −20°C. The precipitate of the acid-soluble proteins was washed twice with ethanol and vacuum dried. ADP-ribosylated histones were separated from unmodified histones by boronate column chromatography as previously described[10].

HPLC of histones

The Beckman HPLC system consisted of two Model 114M pumps, a 421A system controller and a Model 165 variable-wavelength UV–VIS detector. The absorbance of the effluent was measured at 210 nm. The detection signal was recorded by a Shimatzu C-R3A integrator. Histone separations were performed on a Bio-Rad RP-304 C_4 (5-μm silica, 33-nm pore size) column (250 mm × 4.6 mm I.D.). The histone preparations were dissolved in 0.1% TFA. After equilibration of the column with the initial chromatographic solvent, 50 μl of histone solution, containing 225 μg acid-soluble nuclear protein extract, were injected. The histones were chromatographed within 45 min at room temperature and at a flow-rate of 1.3 ml/min. In Fig. 1 a multi-step gradient starting at 74% A–26% B (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile) was used. Successively the concentration of

solvent B was increased linearly from 26 to 31% B (during 5 min), 31 to 33% (10 min), 33 to 38% (5 min), then maintained at 38% (16 min), and increased from 38 to 55% B (15 min). Fractions of 0.65 ml of the HPLC effluent were collected using a fraction collector. To determine the radioactivity of the ^{32}P-labelled proteins, 0.2-ml aliquots were counted in a Beckman liquid scintillation spectrometer.

Electrophoretic analysis of histones

The peak HPLC fractions were appropriately pooled and the solvent vacuum evaporated by the use of a Sera-Vac centrifuge. The samples were analysed in two electrophoretic systems. Sodium dodecyl sulphate (SDS) gel electrophoresis on 15% polyacrylamide slab gels was performed as described by Laemmli[4], except that 4 *M* urea was additionally included in the gel buffer and 6 *M* urea in the sample buffer. These gels were stained with Coomassie Blue R. Alternatively, the samples were electrophoresed on 12% polyacrylamide slab gels (AUT gels) in 0.9 *M* acetic acid–7.5 *M* urea–6 m*M* Triton X-100 as described by Zweidler[5]. The proteins were then detected by amido black staining. The gels were dried and the radioactive compounds detected by autoradiography.

RESULTS AND DISCUSSION

In order to prepare labelled ADP-ribosylated histones, isolated nuclei were incubated with [^{32}P]NAD. As the chromatin-associated nuclear ADP-ribosyltransferase is stimulated by DNA strand breaks, DNase was added to the incubation mixture. Under these conditions approximately 1% of the total histones was ADP-ribosylated. By transfer of the ^{32}P-labelled ADP-ribose moiety of NAD, the acceptor molecules were modified with a single ADP-ribose moiety or a chain of covalently linked residues (for reviews, see refs. 1 and 2). On analysis of the acid extract in the subsequent HPLC experiments, the modified and unmodified histones could easily be differentiated. In the effluent fractions the unmodified histones were detected by their UV absorption and the ADP-ribosylated histones by their radioactivity.

It was our aim to develop an HPLC technique permitting not only good fractionation of individual histones, but also the concomitant collection of specific histones together with their respective ADP-ribosylated derivatives. Preliminary experiments based on our previously developed method[6–8] revealed that shallow gradients gave good fractionation of the unmodified histones. However, this was associated with long elution times, low sensitivity and inconvenient tailing of the ^{32}P-labeled ADP-ribosylated histones. On the other hand, gradients permitting very short elution times gave unsatisfactory separations of the histones. A gradient allowing optimal fractionation of rat liver histones is shown in Fig. 1.

For the identification of the histones in the pooled peak HPLC fractions, two electrophoretic gel systems were used. The AUT gel system had the advantage of satisfactorily separating histone H2A from histone H2B, and of resolving variants of the histones H2A and H3. In both the SDS gel (Fig. 2) and the AUT gel (Fig. 3) systems, the unmodified histones were detected by staining (lane a) and the [^{32}P]ADP-ribosylated histones by autoradiography (lane b). Owing to the different specific activities, various exposure times had to be used in these qualitative analyses of the modified proteins.

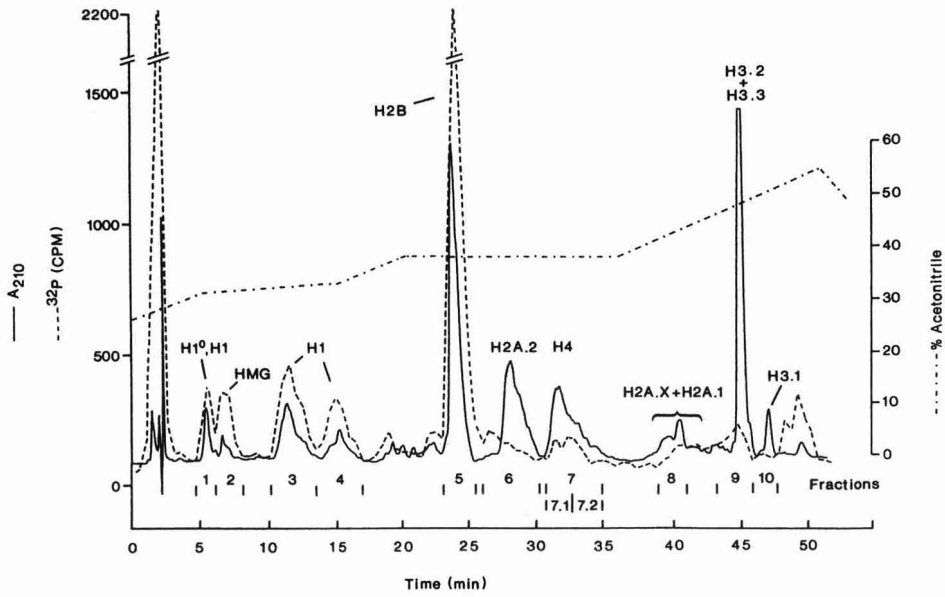

Fig. 1. Fractionation of [^{32}P]ADP-ribosylated and unmodified histones by RP-HPLC. Application of acid-soluble nuclear proteins (225 μg). Other conditions as in Experimental.

According to their position in the HPLC trace (Fig. 1) and their electrophoretic resolution (Figs. 2 and 3), the fractions contained the following proteins: fraction 1, histone H1° and a histone H1 subfraction; fraction 2, probably HMG1 and HMG2 proteins; fraction 3, a histone H1 subfraction; fraction 4, two different histone H1 subfractions; fraction 5, histone H2B; fraction 6, histone H2A.2 and, with increasing mobilities in the AUT gel and lower intensity, probably histone H2A.Z, and oxidized histone H2A.2 (Fig. 3, lane 6a); fraction 7, histone H4; fraction 8, histone H2A.X and histone H2A.1, both discernible in the UV recording of the column effluent and in the gels, histone H2A.X situated at the position of histone H3 in the SDS gel, but above

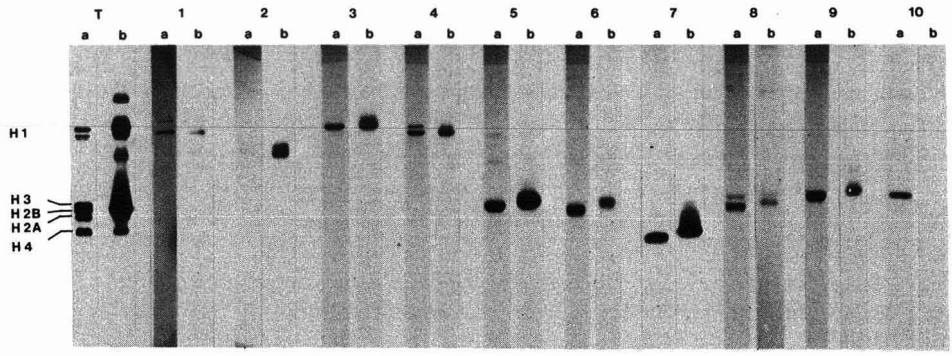

Fig. 2. SDS gel electrophoresis of HPLC fractions. 1–10, Number of effluent fractions, as designated in Fig. 1; T, acid-soluble nuclear proteins applied to the HPLC column. Lanes: a, staining; b, autoradiography.

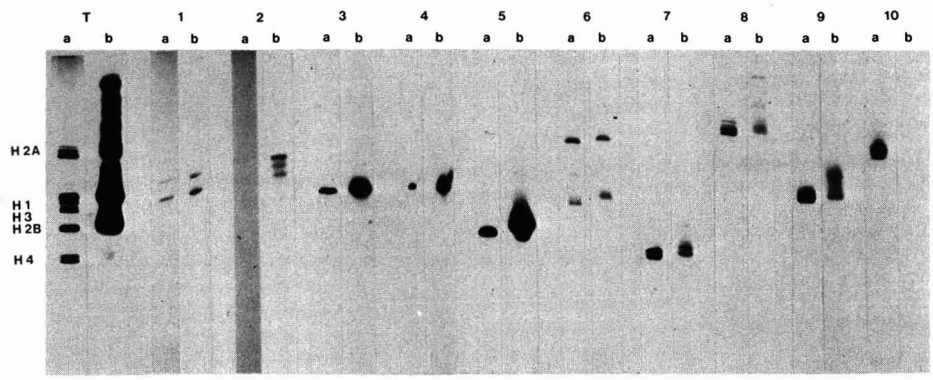

Fig. 3. AUT gel electrophoresis of HPLC fractions. Details as in Fig. 2.

histone H2A.1 in the AUT gel (Fig. 3, lane 8a); fraction 9, a histone H3 subfraction, consisting of the histone H3.2 and histone H3.3 variants; and fracton 10, the histone H3.1 variant.

Additionally, ADP-ribosylated forms of most the above-mentioned proteins were observed. Owing to their low concentrations, the modified proteins were only detectable by autoradiography (Figs. 2 and 3, lanes b). Especially in the SDS gel, the electrophoretic mobilities of the modified proteins were significantly decreased, apparently in accordance with the degree of ADP-ribosylation. This is best illustrated by the case of the fast-moving histone H4 and its derivatives. In accordance with the molecular weight increase, the apparent mono(ADP-ribosyl)ated histone H4 is clearly discernible at a position slightly above that of the unmodified histone (Fig. 2). In addition, a ladder is seen of histone H4 molecules which had been poly(ADP-ribosyl)-ated to different extents, whereas the other histone proteins seem to be mono(ADP-ribosyl)ated.

Moreover, Figs. 1–3 demonstrate that the ADP-ribosylated histones or histone variants were recovered in the same fractions as their unmodified counterparts. No cross-contamination of molecules collected in different HPLC fractions was observed. The recovery of histones and radioactivity was above 85%. Further, Fig. 1 shows that the elution of ADP-ribosylated histones is slightly retarded in comparison with that of the unmodified histones. In essence, however, the introduction of negatively charged ADP-ribosyl residues in the histone molecules and the concomitant molecular weight increase of about 550 Da per monomer unit, did not strongly affect the retention of histones in the HPLC column. Control experiments revealed that the labelled material was sensitive to snake venom phosphodiesterase digestion (not shown).

When material enriched by boronate chromatography was fractionated by means of HPLC, oligo(ADP-ribosyl)ated histones could be detected in all fractions in the autoradiography of the subsequent SDS gel electrophoresis (Fig. 4). Fig. 4 especially demonstrates the advantages of the described method. The autoradiography does not show overlapping zones of modified proteins as observed by the customarily used direct electrophoretic analysis of samples unfractionated by HPLC (Fig. 4, lane T). For example, the ladders of modified histone H2A.2 and H4 molecules clearly depict the modification of the molecules by one to six and one to four,

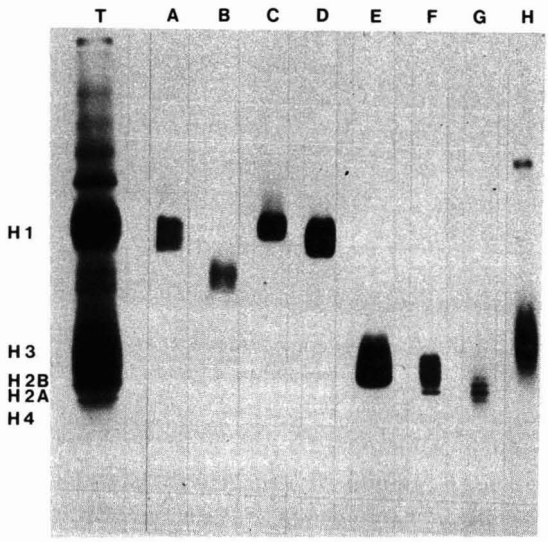

Fig. 4. Analysis of [^{32}P]ADP-ribosylated histones enriched by boronate column chromatography. SDS gel electrophoresis. T, Boronate-enriched material applied to the HPLC column. Collected effluent fractions (corresponding designation in Fig. 1 in parentheses): A, histones H1° and H1 (1); B, HMG proteins (2); C, histone H1 (3), D, histone H1 (4); E, histone H2B (5); F, histone H2A.2 (6); G, histone H4 (7); H, histones H3 (9 + 10).

respectively, ADP-ribose residues. In our experience, the appearence of either ladders or broad bands depends on the combination of certain experimental conditions, such as the degree of labelling, the protein concentration in gel electrophoresis and the exposure time in autoradiography.

In conclusion, using the described HPLC technique ADP-ribosylated histones can be rapidly fractionated for analytical and preparative purposes. Applied solely or in combination with gel electrophoresis, the method will prove useful in studies of ADP-ribosylation reactions that occur under various biological conditions (for reviews, see refs. 1 and 2). The present data illustrate the resolution of several histone H1 subfractions, distinct histone H2A and H3 variants and their respective multiple ADP-ribosyl derivatives.

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Note

Comparison of manual and exponentially modified gaussian based methods for the determination of the peak heights of selected-ion current profiles acquired in a mass spectral drug assay

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(First received August 2nd, 1988; revised manuscript received January 31st, 1989)

The exponentially modified gaussian (EMG) peak shape model[1–14] is widely regarded as giving the most accurate description of chromatographic peaks[15–17]. In particular, this model provides accurate values for chromatographic features of merit such as theoretical plates, asymmetry factors, etc.[10,11]. Surprisingly, no study has been reported on the precision of the model for calculating chromatographic peak heights.

The choice of using peak heights rather than peak areas for quantitative selected-ion monitoring (SIM) measurements has been made over the course of several years' experience with many varied drug assays by ourselves and others[18–20]. The accuracy and precision of peak height measurements exceeds that of peak area calculations, due primarily to interfering compounds eluting close to the analyte of interest and problems in assigning the beginning and end of a peak. The use of peak areas usually requires baseline separation, whereas poorer separations can be tolerated by peak height measurements.

Several years ago, the use of the EMG model to calculate peak heights was incorporated into quantitative selected-ion monitoring processing system (QSIMPS), a collection of hardware and software for the automated collection and analysis of SIM data acquired for use in pharmacokinetic studies[21–23]. First, an estimation of the peak height is done by utilizing a user-chosen baseline along with a quadratic fit of intensity data at the top of the peak. The peak height and retention time estimated from the quadratic fit are then used as initial parameters for fitting to the EMG equation. Briefly, QSIMPS uses NONLIN[24], a popular non-linear regression program, to fit the baseline subtracted ion intensity-time data to the EMG equation shown below:

$$h(t) = \frac{AS}{T}\left(\frac{\pi}{2}\right)^{1/2} \exp\left[\left(\frac{S}{T}\right)^2 \frac{1}{2} - \frac{t-R}{T}\right] \int_{-\infty}^{z2^{\frac{1}{2}}} \frac{1}{(2\pi)^{1/2}} \exp\frac{-y^2}{2}\,dy$$

where $h(t)$ is he peak height at time t, A is the gaussian peak amplitude, S is the standard deviation of the gaussian distribution, T is the time constant of the exponential decay, R is the center of gravity (retention time) of the gaussian peak, and

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$$Z = 2^{-1/2}\left[\frac{(t - R)}{S} - \frac{S}{T}\right]$$

The resulting (returned) values for A, S and R are then used with the time values to calculate a maximum value for the intensity of the peak (peak height). This process is followed for both the analyte peak and the internal standard peak, and the ratio of analyte to internal standard is calculated. The ratio measured in an experimental sample is converted to an amount of analyte using calibration data obtained from the analysis of calibration standards containing various amounts of analyte and a fixed amount of internal standard.

In this note, peak heights of selected ion current profiles determined using the EMG based method used in QSIMPS, are compared with heights determined by manual measurements.

EXPERIMENTAL

The comparison was based on data from a gas chromatographic–mass spectrometric plasma assay for rimantadine[25], an antiviral agent. In this assay, quality assurance samples and calibration standards containing either 500, 200, 50, 20 or 5 ng ml^{-1} of rimantadine were analyzed in duplicate. All samples were fortified with 100 ng ml^{-1} of tetradeuterated rimantadine.

A Carlo Erba gas chromatograph was equipped with a capillary column, Chrompack® CP-Sil 8 CB (25 m × 0.33 mm I.D., film thickness 1.25 μm). The column was maintained at 265°C with methane as the carrier gas. The flow was set to give $1 \cdot 10^{-4}$ Torr source ion gauge reading. The injector, column and interface/transfer line were set to 300, 265 and 300°C, respectively. Under these conditions the retention time of rimantadine was 2.8 min. A Hewlett-Packard Model 7672A automatic liquid sampler was used to inject samples. This auto sampler has a sample capacity of 99 samples.

A Kratos MS-50 magnetic sector mass spectrometer was tuned to give the maximum response consistent with reasonable ion peak shape and a resolution of about 7000. Methane was used as the negative chemical ionization reagent gas. The unlabelled and deuterium-labelled ions were monitored by a Vacuum Generators (VG) digital multiple ion detection (DIGMID) system wired to control the MS 50. These ions were monitored relative to an external lock mass of $C_7F_{14}^-$ (m/z 350) from perfluorotributylamine. The actual ions monitored were the $[M-HF]^-$ ion (m/z 353) of the unlabelled analyte, and the $[M-{}^2HF]^-$ ion (m/z 356) of the tetradeuterated-labelled internal standard.

The output of the DIGMID was sent both to a Lenseis (Princeton Junction, NJ, U.S.A.) eight channel recorder and to QSIMPS. Several recorder channels, set at various attenuations, were connected together so that measurable peaks were obtained for both analyte and internal standard regardless of the response from the sample. The essential components of the QSIMPS hardware consisted of a Hewlett-Packard HP-1000 (A900 processor) with 4.5 Mb of memory, a Model 7937 disk drive with 571 Mb of memory, three HP 3497A interfaces, four HP-2623A terminals, an HP2608S printer with graphics capability, and HP9144A streaming cartridge tape units.

A total of eighteen sets of calibration samples (five calibration concentrations and one quality assurance sample, all analyzed in duplicate) were assayed over a period of several months. The heights of the peaks on the chart paper output of the recorder were measured with a ruler to a resolution of one millimeter. The measured values were manually entered into QSIMPS and were compared with those obtained using the EMG model. Both the manually measured and EMG calculated peak heights for the calibration standards were then regressed against the concentration values. The parameters from the regression analyses were then used to calculate values for the quality assurance sample. The inter-assay precision was estimated from the difference between the observed value for a concentration and the concentration back-calculated from the regression line. The intra-assay precision was estimated from the ratio of the duplicate analyses.

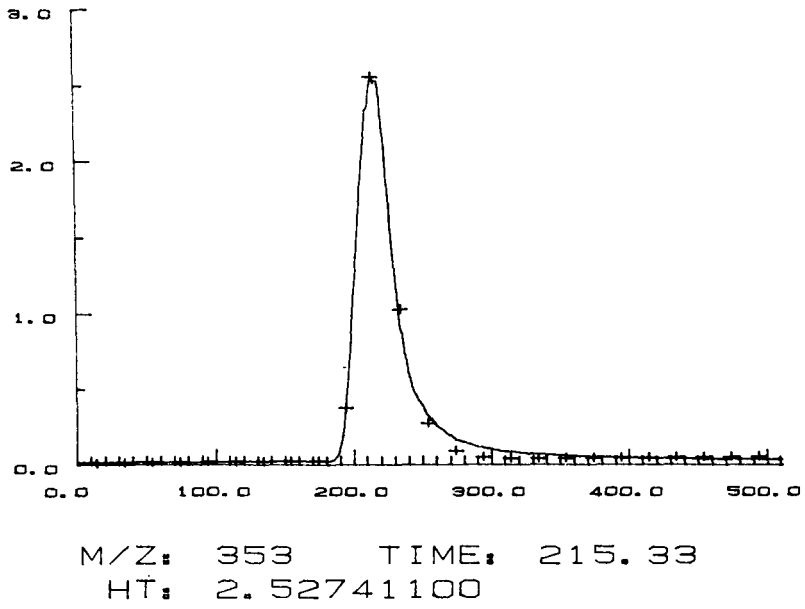
RESULTS AND DISCUSSION

Fig. 1 shows SIM current profiles of the $[M-HF]^{-\cdot}$ ion (m/z 353) from rimantadine and the $[M-{}^{2}HF]^{-\cdot}$ ion (m/z 356) from $[^{2}H_4]$-rimantadine. The solid line represents the raw data, actually made up of 512 data points over the retention time window shown. The crosses represent the EMG fit using the top 95% of the peak. The baseline was chosen by extrapolation between the average voltage from scans 150–160 and scans 400–410. Note that the calculated peak height and the peak height from the SIM chromatogram are nearly identical. Also, since only the top 95% of the peak was used for the fit, note that the calculated peak tail is slightly less than the actual SIM profile.

Data from the comparison are given in Table I. Both methods of peak height measurement gave similar results, *i.e.*, a linear regression analysis of back-calculated concentrations measured either by hand (y) or by the EMG-based method (x) gave a slope of 1.006 and an intercept of 0.76 ng ml^{-1}. The correlation coefficient for the regression was 0.9999.

Almost without exception, the comparison of the data from both methods of peak height measurement showed that the EMG based method gave superior results. Compared with the manual measurements, the inter-assay precision from the EMG based measurements was lower at every calibration concentration and for the quality assurance sample. The mean improvement in relative standard deviation was 16%. Compared with the manual measurements, the intra-assay precision from the EMG based measurements was lower at three of the five calibration concentrations and for the quality assurance sample. The mean improvement (including the two concentrations not showing improvement) was approximately 5%.

The EMG model for chromatographic peaks has not previously been incorporated into an on-line data system. This model is valued by professional chromatographers because it accurately accounts for the internal and extracolumn processes responsible for peak tailing. However, to the ordinary chromatographer, the essential citerion for any chromatographic peak model is whether it improves the precision of the collected data. In this regard, SIM current profiles are a good test of any model because of the high degree of noise associated with such measurements. The results reported here suggest that the EMG model yields a more precise determination of the peak heights than hand measurements. In addition, it should be noted that, although



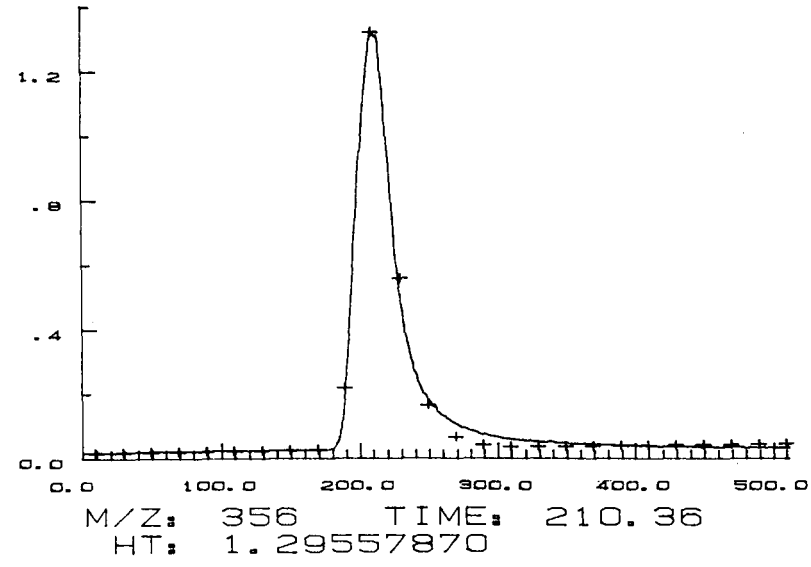


Fig. 1. Selected-ion monitoring current profiles of the $[M-HF]^{-\cdot}$ ion (m/z 353) from rimantadine and the $[M-{}^{2}HF]^{-\cdot}$ ion (m/z 356) from $[^{2}H_4]$rimantadine. The raw data is represented by the solid line and the crosses represented the calculated EMG fit using the top 95% of the peak.

TABLE I

COMPARISONS OF DATA FROM THE ANALYSIS OF THE SAME SAMPLE WITH PEAK HEIGHTS DETERMINED BY MANUAL AND EMG-BASED METHODS

Data were collected and processed as described in the Experimental section. Data in parentheses are for the EMG-based measurements. Data from manual measurements are without parentheses.

Sample[a]	*Inter-assay precision (found concentration ± S.D.*[b]*, R.S.D.*[c]*)*	*Intra-assay precision (ratio*[d] *± S.D., R.S.D.)*
500 ng/ml	496 ± 12, 2.3% (492 ± 9.0, 1.8%)	0.98 ± 0.06, 6.1% (0.98 ± 0.04, 4.1%)
200 ng/ml	202 ± 12, 5.8% (206 ± 9.0, 4.4%)	0.99 ± 0.06, 6.0% (1.01 ± 0.06, 6.0%)
50 ng/ml	53 ± 3.9, 7.4% (54 ± 3.9, 7.2%)	1.00 ± 0.04, 4.0% (1.00 ± 0.04, 4.0%)
20 ng/ml	21 ± 2.0, 9.2% (21 ± 1.5, 7.1%)	1.05 ± 0.11, 10% (1.02 ± 0.09, 9.0%)
5 ng/ml	4.3 ± 0.9, 21% (4.3 ± 0.8, 19%)	1.00 ± 0.22, 22% (0.97 ± 0.25, 26%)
Quality assurance	63 ± 5.5, 8.7% (63 ± 3.1, 5.0%)	1.00 ± 0.09, 9% (1.01 ± 0.05, 5.0%)

[a] Eighteen calibration curves (five calibration standards and one quality assurance sample, all analyzed in duplicate).

[b] S.D. = Standard deviation.

[c] R.S.D. = Relative standard deviation.

[d] Ratio of back-calculated concentration from first determination of the duplicate pair divided by back-calculated concentration from second determination.

the SIM current profiles used in this comparison had excellent signal-to-noise characteristics, the chromatographic conditions varied significantly over the course of the experiment, demonstrating that the EMG model can accurately describe both sharp and tailing peaks.

Most mass spectrometer data systems determine peak heights of SIM current profiles by essentially automating conventional manual methods, *i.e.*, finding an appropriate baseline and peak, and assigning a peak height by finding the maximum voltage for the peak. This process can easily lead to inaccurate results when the peaks have low signal-to-noise ratios because the data system will often assign a voltage maximum which corresponds to a noise spike. Although, these noise spikes and other irregularities can be easily recognized visually, peak height determinations based on manual measurements were still found to be less precise then EMG based measurements.

Model based peak height measurements offer potential advantages in chromatographic assays. When the analyte being measured is known, the parameters describing the shape of the analyte peak in an experimental sample can be compared with the parameters from a stable isotope internal standard in order to verify compound identification. For example when an electronic noise spike occurs in the retention time window of interest, it is desirable that the peak be disregarded. One way to do this is to compare the peak fitting parameters of the stable isotope reference standard to that of the noise spike. In most cases, the peak width of the noise spike is different than that of the authentic standard and therefore the spike would not be identified as the analyte peak. This capability is not currently being applied in our laboratory but is a possible application of model based peak height measurements. Model based characterizations are a starting point for the greater use of the increased

computational capacity now available to an analyst to interpret and characterize chromatographic data. In this regard, the EMG model is still a relatively unrefined representation of the phenomena occurring in a chromatographic column. The EMG model will certainly be succeeded by more accurate expressions which, based on the results reported here, should yield even more precise peak height measurements.

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Note

Altered retention of derivatives of *tele*-methylimidazoleacetic acid in acid-hydrolyzed samples of brain measured by capillary gas chromatography and mass spectrometry

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Classical chromatographic science holds that chemical substances, even those of similar structure, can be separated according to each compound's independent interaction with chromatographic media. In gas chromatography (GC), the hallmark of chromatographic separation of a substance is its characteristic retention, often expressed as a retention time, highly reproducible under defined conditions of column composition, size, gas flow, temperature, etc. With capillary GC columns, the range and variability of characteristic retention times are extremely narrow. Significant deviations from established retention times are uncommon when measured under identical chromatographic conditions[1,2]. Yet it has been observed, for instance, that if a trace constituent elutes after a much larger component[3], the latter acts as a transient stationary phase, increasing retention of the former. Absent such phenomena, in capillary GC analysis it is rare[1,2,4] to observe selective deviations from well characterized retentions of substances due to component(s) of the injectate under otherwise identical conditions. Under identical GC conditions we observed that the retention times of both derivatized *tele*-methylimidazoleacetic acid (t-MIAA) and its deuterated form ([$^2H_3$]t-MIAA) were consistently and reversibly increased. This occurred in samples prepared from homogenates of rat brains that were hydrolyzed but not in those prepared from aliquots of the same homogenates kept at room temperature or frozen. We present results of a typical experiment, extended briefly to study this effect.

EXPERIMENTAL

Male Sprague-Dawley rats (200–400 g) were anesthetized with methohexital sodium (75 mg/kg, intraperitoneal), underwent cerebral perfusion[5] to remove blood, and were decapitated. Whole brains were homogenized with 4 vol. of distilled water. Aliquots were mixed with equal volumes of 0.2 *N* hydrochloric acid, vortexed, boiled for 10 min, cooled, then stored frozen at −80°C. Later, the acidified, boiled homogenates were thawed and vortexed, then several 1-ml aliquots were transferred to glass vials containing 1 ml 0.1 *N* hydrochloric acid and internal standards for t-MIAA and imidazoleacetic acid (IAA) (see below). Some samples were transferred to

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hydrolysis tubes (Pierce) then heated at 150°C; others, from the same homogenates, were kept at room temperature. After 72 h, hydrolyzed and non-hydrolyzed samples were transferred to polypropylene tubes and centrifuged at 50 000 *g* for 20 min. Supernatants were retained for analysis.

t-MIAA was extracted, separated by ion-exchange chromatography, derivatized with boron trifluoride–butanol and measured by GC–mass spectrometry (MS) by the method of Khandelwal *et al.*[6] as modified[7]. In the same samples, IAA was extracted and its acidic side chain was derivatized along with t-MIAA. After mixing derivatized IAA with ethyl-chloroformate, *n*-butyl-(N-ethoxycarbonyl)imidazoleacetate was produced; two isomeric compounds are formed since IAA has two tautomeric forms[5]. Samples were injected onto a Durabond fused-silica capillary column [DB-WAX, polyethylene glycol phase with 0.25-μm phase film thickness; 15 m × 0.25 mm I.D. (J. & W Scientific)]. Derivatives of t-MIAA, its internal standard, [$^2H_3$]t-MIAA, and IAA and its internal standard, ^{15}N,^{15}N-IAA, were analyzed by methane chemical ionization (CI) dual ion-group monitoring on a Hewlett-Packard combined gas chromatograph (HP 5890)–mass spectrometer (HP 5988A) interfaced with an HP 59970B workstation. Selected mass ions (*m/e*) were monitored from 7 to 9 min after injection of 3 μl of sample. Samples were chromatographed in the slitless mode with helium (10 p.s.i. head pressure) as carrier gas. The methane pressure was 1 Torr in the ionization source. The oven temperature was initially equilibrated at 100°C, then ramped at 30°C/min after sample injection, up to 200°C. Injection port, gas chromatograph–mass spectrometer transfer line and ion source were maintained at 250, 250 and 150°C, respectively. For IAA and ^{15}N,^{15}N-IAA, the larger of each of the two peaks of the isomers was evaluated[5]. Authentic standards (0.1–150 ng of t-MIAA and IAA) in 0.1 *N* hydrochloric acid were processed in parallel. We compared retention times since all other aspects of the analyses were held constant. Differences among means of retention times, determined at maximal height, were evaluated by analysis of variance (ANOVA). Differences between groups were assessed using Dunnetts multiple range test.

RESULTS AND DISCUSSION

The retention times of derivatized t-MIAA (*m/e* 197) and IAA (*m/e* 255) and their respective internal standards (*m/e* 200 and 257) from the various sample groups are shown in Table I. In samples that had been hydrolyzed, the retention times of derivatives of t-MIAA and [$^2H_3$]t-MIAA were altered, each to the same extent; the retention times of derivatives of IAA and ^{15}N,^{15}N-IAA in the same injectates were unchanged. Although the mean retention time of derivatized t-MIAA in hydrolyzed samples was increased only 3% compared to retention of non-hydrolyzed samples, this shift was highly significant (ANOVA: $p < 0.0001$), representing a mean peak prolongation of about 10 s. The 95% confidence interval range about the mean retention times of hydrolyzed and non-hydrolyzed samples did not overlap and none of the retention times of derivatized t-MIAA from hydrolyzed samples was within the confidence range of samples kept at room temperature; the latter were almost identical to values for authentic standards (Table I). In each hydrolyzed sample, the retention time of derivatized t-MIAA always exceeded that of its non-hydrolyzed sample pair. Changing the order of injections or alternating between samples prepared from

TABLE I

RETENTION TIMES OF DERIVATIVES OF t-MIAA (*m/e* 197), [$^2H_3$]t-MIAA (*m/e* 200), IAA (*m/e* 255) AND ^{15}N,^{15}N-IAA (*m/e* 257) OF HYDROLYZED AND NON-HYDROLYZED SAMPLES MEASURED BY GC–MS

Arithmetic means ± S.E.M. of 11–20 independent replicates.

	Retention time (min) (mean ± S.E.M.)			
	m/e 197	*m/e 200*	*m/e 255*	*m/e 257*
Authentic	7.290 ± 0.010	7.285 ± 0.010	8.337 ± 0.003	8.337 ± 0.003
Non-hydrolyzed	7.270 ± 0.005	7.264 ± 0.005	8.328 ± 0.007	8.328 ± 0.007
Hydrolyzed	7.439 ± 0.027[a]	7.424 ± 0.027[a]	8.352 ± 0.014	8.345 ± 0.012
Hydrolyzed and dried authentic	7.378 ± 0.017[b]	7.368 ± 0.018[b]	8.357 ± 0.003	8.364 ± 0.004
Hydrolyzed mixed with authentic	7.347 ± 0.008[c,d]	7.338 ± 0.011[c,d]	8.310 ± 0.002	8.310 ± 0.002
ANOVA				
F	18.27	14.98	1.59	2.16
p	<0.0001	<0.0001	Not significant	Not significant

[a] $p < 0.001$ *versus* authentic and non-hydrolyzed.
[b] $p < 0.01$ *versus* authentic and non-hydrolyzed.
[c] $p < 0.05$ *versus* authentic and non-hydrolyzed.
[d] $p < 0.05$ *versus* hydrolyzed only.

hydrolyzed or non-hydrolyzed material or authentic compounds did not influence the retention time or area counts of analytes within each group. The aqueous standards were unaltered after acid hydrolysis. There was no correlation between the retention time and the magnitude of the elevation[8] of the levels of t-MIAA or IAA in hydrolyzed homogenates. There was no evidence for extra peaks or tailing in any of the preparations. Mass fragmentography confirmed the identity[5,6] of the derivatives of t-MIAA (evaluated at *m/e* 95), IAA (evaluated at *m/e* 81, 154 and 254) and their internal standards at the various retention times.

Prolonged retention of a constituent due to the components of a sample has been known to occur with column overloading (*e.g.* ref. 3). Retention of trace substances may be retarded when eluted after a major component. This produces broadening of the major peak but not of the minor peak; the latter is unsymmetrical, and elutes earlier as sample size increases[3]. However in our samples, total ion scanning of hydrolyzed and non-hydrolyzed material during the first 10 min after sample injection indicated that no major peaks (*i.e.* those greater than 10% of area counts of t-MIAA) eluted within 2 min before the peak for t-MIAA. Injection of larger samples slightly increased the retention times for the t-MIAA derivatives. Moreover in the hydrolyzed samples, peaks of the derivatives for t-MIAA and [$^2H_3$]t-MIAA were symmetrical. The peaks ($n = 19$) for derivatized t-MIAA in hydrolyzed samples, evaluated by valley-to-valley baseline analysis on the system's software, were an average of 3.78 s broader ($p < 0.001$) than peaks from samples kept at room temperature, whose average peak width was 5.12 ± 0.29 (S.E.M.) s. Thus, there was no evidence for a single hydrolysis component acting as a stationary phase in our study. This suggested a different mechanism that

affected the elution properties of the derivatives of t-MIAA, but not those of IAA, from Durabond columns.

To probe this phenomenon further, hydrolyzed samples that had been analyzed were diluted with chloroform and spiked with aliquots prepared from either 30 or 100 ng of authentic t-MIAA and IAA. Authentic material was transferred to silanized glass vials and either (a) evaporated to dryness then resuspended with 5 μl of hydrolyzed sample, or (b) mixed with an equal volume of hydrolyzed sample. Under both circumstances, the retention times of derivatives of t-MIAA and [$^2H_3$]t-MIAA, but not those of IAA or its internal standard, were significantly and consistently increased compared to non-hydrolyzed samples or standards in aqueous media (Table I). The increased retention was independent of the quantity of authentic compound. The mean retention time of dried standards resuspended with hydrolyzed samples did not differ significantly from mean values of the latter. The retention times of standards mixed in equal volume with hydrolyzed samples, which diluted the latter, were midway between, and differed significantly (each $p<0.05$) from, the retention times of both non-hydrolyzed and hydrolyzed samples (Table I). Therefore, as the fraction of hydrolyzed material in the injectate decreased, so did the retention time for t-MIAA. A similar but lesser shift of the retention time was observed in hydrolyzed samples of cerebrospinal fluid[9]; the latter samples presumably contain fewer components than tissue homogenates contain. Since it is likely that amounts of chloroform in sample vials vary to some degree, this may partially account for the greater variation in retention values from hydrolyzed samples. Consonant with this hypothesis, the coefficient of variation from the latter was about five-fold higher than that of non-hydrolyzed samples.

The precise reason(s) for this selective change in the retention time is unknown. Non-specific factors such as increased ionic strength and reduced lipid composition in these hydrolyzed samples probably have little bearing since the retention of the IAA derivatives was unchanged. Nevertheless, it is apparent that components in the injectate prepared from hydrolyzed homogenates of rat brain have a significant influence on the elution characteristics of t-MIAA derivatives but not on IAA derivatives on this and other Durabond-wax columns (obtained from the same manufacturer) that we have used. Since the side chains of derivatized t-MIAA and IAA are identical[5,6], the column–analyte interaction(s) responsible for these changes most likely rest with the substituted nitrogen of the imidazole moiety of the t-MIAA molecule. Such an interaction(s) is not immediately obvious since the rings for t-MIAA and derivatized IAA have no reactive nitrogens, the former is methylated, the latter is linked to a carboxyethyl group.

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Note

Sulphated Sepharose —a strong cation exchanger

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Cellulose-, silica- and Sephadex-based ion exchangers are frequently used for the separation of large molecules. During the last decade, ion exchangers based on other carriers such as Sepharose have also become commercially available. In this paper we report a simple method for preparing cation exchangers by sulphation of cross-linked polysaccharides, developed about 15 years ago but which we believe is still useful. The preliminary characterization of a sulphated Sepharose product and a comparison with the capacity of the commercially available cation exchangers[1] SE-Sephadex C-25[a] and SP-Sephadex C-50 are described.

EXPERIMENTAL

Sepharose 6B and 2B, Sephadex G-50, SE-Sephadex C-25 and SP-Sephadex C-50 were obtained from Pharmacia (Uppsala, Sweden). Epichlorohydrin cross-linked Sepharose (ECD-Sepharose) was prepared as described by Porath *et al.*[2]. Sulphur trioxide and pyridine were purchased from Kebo (Stockholm, Sweden), cytochrome *c* from Serva (Heidelberg, F.R.G.) and *Phaseolus vulgaris* (red kidney beans, type Stella Y/500) from Weibullsholm (Landskrona, Sweden). The agglutination activity (titre) was measured according to Salk[3] using a 2% phosphate-buffered saline suspension of three-times washed human red cells of A-type.

Preparation of sulphur trioxide–pyridine complex

A three-necked flask containing 150 ml of pyridine was equipped with a thermometer, a reflux condenser, a dropping funnel, and placed in an ice-bath on a magnetic stirrer. A volume of 20 ml of sulphur trioxide was added to the chilled pyridine, dropwise and with vigorous stirring. After the completed reaction, the flask was stoppered and the product was stored at 5°C.

Sulphation reaction

A 20-ml volume of the gravity-settled ECD-Sepharose 6B was washed on a glass filter funnel with 200 ml of distilled water, 400 ml of ethanol and 400 ml of pyridine.

[a] From January 1970 SP-Sephadex replaces the earlier SE-Sephadex. The two types have very similar properties (see Ref. 1, footnote to Table I).

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The suction-dried material was transferred into a 100-ml flask by means of a few millilitres of pyridine and allowed to swell. After addition of 20 ml of the suspension of the sulphur trioxide–pyridine complex, the flask was stoppered and the mixture was gently shaken at room temperature overnight. The sulphation of ECD-Sepharose 2B and of Sephadex G-50 was achieved in a similar way. The resulting product was washed on a glass filter funnel with about 0.6 l each of distilled water, ethanol, 1 *M* acetic acid containing 2 *M* sodium chloride, 1 *M* sodium carbonate containing 2 *M* sodium chloride and, finally, distilled water. For sulphur analyses[4,5], about 1 ml of the product was washed on a small glass filter funnel with 20 ml of ethanol and 20 ml of acetone and was lyophilized. The products were kept swollen in 0.1 *M* ammonium formate buffer (pH 3) containing 1 *M* sodium chloride and 0.1% sodium azide. Equilibration and regeneration were carried out according to the manufacturer's instructions[1] for equilibration and regeneration of SP-Sephadex (counter ion: ammonium).

Titration

Potentiometric titrations were performed principally as described by Porath and Fornstedt[6]. A gel volume sufficient to give at least 5 ml of sediment was allowed to settle in a cylinder and 5 ml of the sediment (corresponding to about 0.4 g of sulphated ECD-Sepharose 6B) were washed on a glass filter funnel with 50 ml of 0.5 *M* hydrochloric acid and then with 0.5 l of distilled water. The suction-dried material was transferred into a laboratory-made titration vessel[6] and 25 ml of 1.0 *M* sodium chloride were added. After adjusting of pH to below 2.0 with a few drops of 1.0 *M* hydrochloric acid, titration was effected by 0.05-ml portions of 0.1 *M* sodium hydroxide solution.

Preparation of kidney-bean extract

The extraction technique described by Rigas and Johnsson[7] was used with some modification. A 1-kg amount of finely ground red beans was extracted at pH 1 overnight with 5 l of 0.1 *M* hydrochloric acid. After readjusting the pH, the suspension was centrifuged and the clear supernatant dialysed against 0.1 *M* ammonium formate buffer (pH 4). The inactive precipitate was removed by centrifugation and the clear liquid lyophilized, giving about 35 g of material.

Adsorption capacity

Three columns of 3 cm × 10 mm I.D. were packed with the ion exchangers SE-Sephadex C-25, SP-Sephadex C-50 and sulphated ECD-Sepharose 6B. The available capacity for cytochrome *c* and kidney-bean proteins was determined by frontal analysis as follows. The packings were equilibrated with 0.1 *M* ammonium formate buffer (pH 3). A solution of 1 mg/ml of cytochrome *c* in the buffer was continuously pumped through at a flow-rate of 10 ml/h until the effluent showed the same absorbance (A_{254}) as the introduced solution. After thoroughly washing with the buffer, the adsorbed material was eluted with 0.1 *M* ammonium formate buffer (pH 8). UV-positive desorption fractions were pooled, concentrated and lyophilized. The available capacity for the kidney-bean proteins was determined as described for cytochrome *c* with the following modifications: two columns of 8 cm × 10 mm I.D. were packed with the SP-Sephadex C-50 and sulphated ECD-Sepharose 6B and the pack-

TABLE I

SULPHUR CONTENTS OF SULPHATED PRODUCTS

Product	*S (mg/g)*[a]	*Total capacity*[b] *(mequiv./g)*[a]
Sulphated ECD-Sepharose 6B	149	4.6[c]
Sulphated ECD-Sepharose 2B	114	3.5
Sulphated Sephadex G-50	140	4.4

[a] Dry weight.

[b] For the total capacities of the sulphopropyl substituted Sephadex types, see Table II in Ref. 1.

[c] To be compared with 4.1 mequiv./g as calculated from the titration curve (Fig. 1).

ings were equilibrated with 0.1 *M* ammonium formate buffer (pH 4) and saturated with a solution of 10 mg/ml of lyophilized kidney-bean extract in the starting buffer.

RESULTS AND DISCUSSION

The sulphur concentrations of the products are given in Table I. The capacity of the sulphated ECD-Sepharose 6B was almost constant over a fairly wide pH range (Fig. 1). As shown in Table II, the rate of the sulphating reaction is highest within the first 5 min. Likewise, a 2-h incubation time provides 80% yield. The sulphated gels withstood exposure to strongly alkaline conditions, which is of importance for the regeneration procedure. Sulphated ECD-Sepharose 6B seems to possess a higher capacity for small than for larger proteins and to show properties similar to those of SE-Sephadex C-25 with respect to small proteins (Table III). Sulphated products of the cross-linked polysaccharides are strongly acidic cation exchangers; the functional groups consist of monovalent ions.

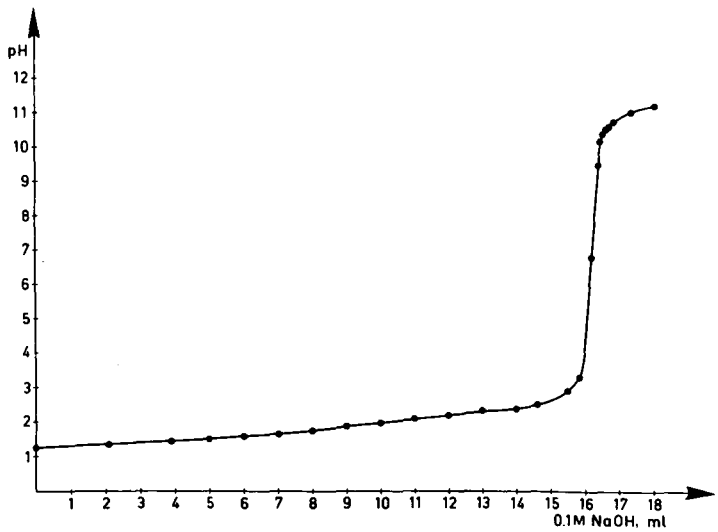

Fig. 1. Titration curve for sulphated ECD-Sepharose 6B.

TABLE II

EFFECT OF INCUBATION TIME ON THE SULPHATION OF ECD-SEPHAROSE 6B

Incubation time (min)	*S (mg/g)*[a]
0	0.4
5	60.5
15	77.0
30	89.5
120	128.0
900	151.0

[a] Dry weight.

TABLE III

AVAILABLE CAPACITY

Ion exchanger	*Cytochrome c (mg/ml)*	*Red bean proteins (mg/ml)*
Sulphated ECD-Sepharose 6B	260	220[a]
SE-Sephadex C-25	270	–
SP-Sephadex	40	940[b]

[a] Total titre: 4300.
[b] Total titre: 18800.

The procedure for the preparation of the sulphur trioxide–pyridine complex is simple. The sulphating reaction and the equilibration stage are not laborious and do not require the use of special equipment. As can be seen in Table I, the general approach can also be utilized for the sulphation of cross-linked dextrans. Further, desorption with concentrated salt solutions does not cause any shrinking. Moreover, the possibility of preparing ion exchangers with different matrices, porosities and ionic densities offers flexibility in the choice of the most suitable ion exchanger for an appropriate separation problem.

ACKNOWLEDGEMENTS

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CHROM. 21 414

Note

Direct resolution of anthelmintic drug enantiomers on Chiral-AGP protein-bonded chiral stationary phase

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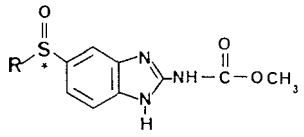
Albendazole (ABZ, Zental® or Valbazen®) and fenbendazole (FBZ, Panacur®) are benzimidazole anthelmintic drugs, used in the treatment of helminthiases in animals[1] and man[2]. After oral administration, the sulphide (ABZ or FBZ) is oxidized to the corresponding sulphoxide SOABZ (or SOFBZ)[3,4], which bears an asymmetric sulphur centre (Fig. 1). The sulphone compound SO_2ABZ (or SO_2FBZ) is also present as a metabolite. The sulphoxide is considered to be responsible for the anthelmintic activity; however, apart from this therapeutic property, embryotoxicity has been found in rats[5,6].

The liquid chromatographic (LC) separation of SOABZ and SOFBZ enantiomers on chiral stationary phases (CSPs) will provide a useful means of studying stereoselectivity in the pharmacokinetic disposition of these metabolites in animals and man. The resolution of SOABZ and SOFBZ enantiomers has been described previously on a CSP synthesized from (*S*)-N-(3,5-dinitrobenzoyl)tyrosine as chiral selector[7]. A baseline resolution of SOABZ enantiomers was achieved allowing enantiomeric assays of these metabolites in human, bovine, sheep and rat plasma samples[7]. Differences in the enantiomeric ratios were found, depending on the species,



SOABZ R = $-(CH_2)_2-CH_3$

SOFBZ 

Fig. 1. Structures of albendazole sulphoxide (SOABZ) and fenbendazole sulphoxide (SOFBZ).

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which could be attributed to different enzymatic processes. However, the resolution of SOFBZ enantiomers was poor (resolution factor $R_s \leqslant 0.8$) and still remained a challenge.

The use of bovine serum albumin (BSA) bonded phase (Resolvosil-BSA-7 column, available from Macherey, Nagel & Co., Düren, F.R.G.) has been described by Allenmark *et al.*[9] for the resolution of pharmacologically active chiral sulphoxides containing a benzimidazole moiety. This paper reports the direct LC resolution of SOABZ and SOFBZ enantiomers on a commercially available CSP, Chiral-AGP (ChromTech, Stockholm, Sweden) developed by Hermansson[10] and Enquist and Hermansson[11], and for which plasma α_1-acid glycoprotein is immobilized according to a novel technique on spherical porous silica particles ($d_p = 5$ μm).

EXPERIMENTAL

Apparatus

Analytical chromatography was performed with a modular liquid chromatograph (Gilson, Villiers-le Bel, France) equipped with a Model 802C manometric module, a Gilson 811 (1.5-ml) dynamic mixer and a Model 116 variable-wavelength UV detector. The column and solvent were thermostated with a Haake Model D8-V circulator bath (-5 to 150°C) (Roucaire, Vélizy-Villacoublay, France) and a water cooling-jacket. All tubing connections were heat-insulated.

Chiral stationary phases

A 100 × 4.0 mm I.D. Enantiopac column was purchased from LKB (Les Ulis, France). The plasma protein α_1-AGP was immobilized on a 10-μm diethylaminoethylsilica gel by ionic bonding followed by cross-linking. A Chiral-AGP (100 × 4.6 mm I.D.) column was purchased from ChromTech. In both instances t_0 was measured by injection of methanol.

Solutes and solvents

Samples of ABZ and FBZ sulphides, the corresponding SOABZ and SOFBZ sulphoxides (as racemates) and SO_2ABZ and SO_2FBZ sulphones were donated by Professor P. Delatour (Laboratoire de Biochimie, I.N.R.A. 54189, Ecole Nationale Vétérinaire de Lyon, France).

Aqueous buffer solutions were prepared from sodium phosphate buffer (25 m*M*, pH 6.88) purchased from Merck (Darmstadt, F.R.G.). Deionized water was doubly distilled on a Büchi-Fontavapor 285 apparatus (Roucaire). The pH of the aqueous buffer eluent was controlled with a Model Minisis 8000 pH/millivoltmeter (Tacussel, Villeurbanne, France) and Tacussel glass TB/HS and Tacussel C8 calomel reference electrodes. Aqueous solvents were filtered through 0.65-μm Type DAWP Millipore membrane filters (Touzart et Matignon, Paris, France) and then degassed with helium. 2-Propanol was of LiChrosolv grade purchased from Merck.

Solutes were first dissolved in 2-propanol in an ultrasonic bath; the solution was then diluted 5-fold with 8 m*M* sodium phosphate buffer (pH 7.0). The concentrations of the solutes were chosen around $3 \cdot 10^{-2}$ mg ml^{-1}, corresponding to an amount injected of about 2 nmol (20 μl)

RESULTS AND DISCUSSION

As shown in Fig. 2 for the resolution of SOFBZ enantiomers, the Chiral-AGP column displays a higher efficiency than the Enantiopac column. Moreover, shorter analysis times are afforded, as the Chiral-AGP column can be used without flow restrictions.

The retention and enantioselectivity (α) are highly affected by the concentration of 2-propanol in the mobile phase, as demonstrated in Table I. A decrease in the 2-propanol content from 2% to 0% resulted in a strong improvement in the enantioselectivity and an increase in retention. This is in accordance with previous findings for other classes of compounds[10–16]. The enantiomers of SOFBZ are, therefore, completely resolved using a mobile phase without 2-propanol (Fig. 3). The organic modifier probably competes with the uncharged solute for binding to the protein, as previously suggested by Hermansson and co-workers[11,12,16], through hydrophobic and/or hydrogen bonding interactions. The stereospecific binding of the chiral drug to the binding sites of α_1-acid glycoprotein may be altered in the presence of 2-propanol owing to conformational changes in the flexible protein structure.

From the chromatogram in Fig. 2B, it is obvious that a separation factor of *ca.* 1.25 is insufficient to obtain a complete baseline resolution of SOFBZ. However, a complete resolution ($R_s \geqslant 1.5$) of SOFBZ is achieved using a mobile phase without 2-propanol (Fig. 3). Under the same mobile phase conditions, SOABZ enantiomers

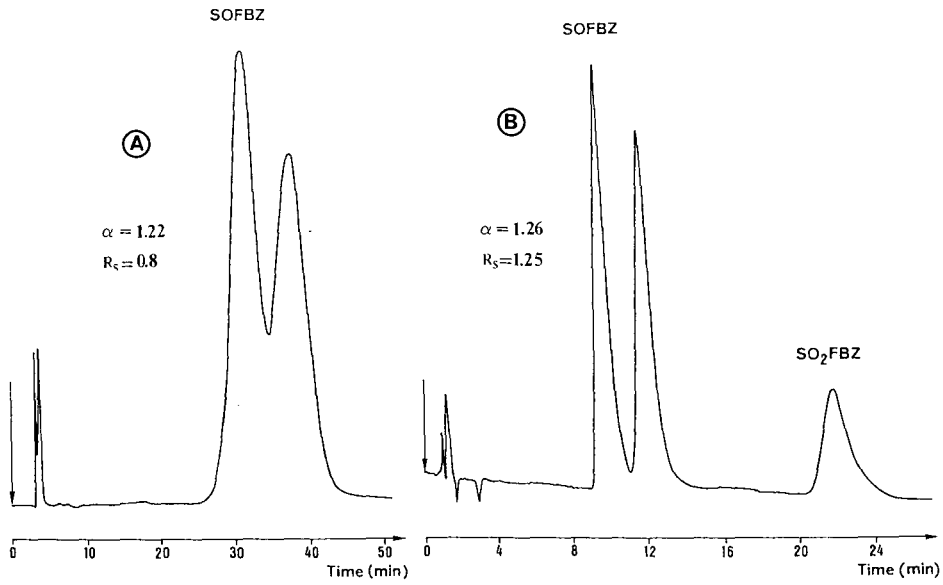

Fig. 2. Comparison of resolution of oxfendazole enantiomers (SOFBZ) on two acid α_1-glycoprotein bonded columns. (A) Enantiopac column (d_p = 10 μm); mobile phase, 8 m*M* sodium phosphate buffer (pH 7.0) + 0.1 m*M* NaCl + 2% (v/v) 2-propanol; flow-rate, 0.3 ml min^{-1}. (B) Co-injection of SOFBZ and SO_2FBZ on a Chiral-AGP column (d_p = 5 μm); mobile phase, 8 m*M* sodium phosphate buffer (pH 7.0) + 2% (v/v) 2-propanol; flow-rate, 0.9 ml min^{-1}; temperature, 25°C; UV detection at 220 nm. Efficiencies measured by means of reduced plate height, $h = L/Nd_p$: (A) $h \approx 40$, $k'_2 = 10.0$; (B) $h \approx 20$, $k'_2 = 10.8$.

TABLE I

INFLUENCE OF THE ADDITION OF 2-PROPANOL TO THE AQUEOUS MOBILE PHASE ON THE RESOLUTION OF SOABZ AND SOFBZ ENANTIOMERS AND ON THE RETENTION OF SO_2ABZ AND SO_2FBZ SULPHONES

Column, Chiral-AGP; mobile phase, 8 m*M* sodium phosphate buffer (pH 7) with 2-propanol added; flow-rate, 0.9 ml min^{-1}; temperature, 25°C; UV detection at 220 nm.

2-Propanol (%,v/v)	*SOABZ*			*SO_2ABZ, k'*	*SOFBZ*			*SO_2FBZ, k'*
	k'_2[a]	α[b]	R_s[c]		k'_2	α	R_s	
2	2.70	1.35	1.1	2.69	10.80	1.26	1.1	20.6
1	3.97	1.51	1.25	3.70	14.23	1.28	1.25	
0	7.33	1.68	1.4	6.37	18.94	1.54	1.6	

[a] The capacity factor k'_2 (of the second eluted enantiomer) was calculated as follows: $k'_2 = (t_{R2} - t_0)/t_0$.

[b] Selectivity $\alpha = k'_2/k'_1$.

[c] R_s (resolution factor) = 2 (distance of the two enantiomer peak positions/sum of the band widths of the two peaks at their bases); $R_s = 2(t_{R2} - t_{R1})/(w_1 + w_2)$.

are almost baseline resolved, despite the peak asymmetry of the first eluted enantiomer (Fig. 4).

SOFBZ enantiomers are more retained than the SOABZ enantiomers; enhancement of hydrophobic interactions with the protein caused by the phenyl group of SOFBZ may be responsible.

The capacity factors of the sulphone compounds are also given in Table I and demonstrate that SO_2ABZ is eluted between the two enantiomers of SOABZ (Fig. 4). This constitutes a drawback for accurate assays of SOABZ enantiomers in biological samples containing the sulphone metabolite. Fortunately, such peak overlapping does not occur with SOFBZ and SO_2FBZ (Fig. 2B).

The above findings demonstrate the chiral recognition ability of Chiral-AGP towards a new family of anthelmintic drugs. However, further investigations will be

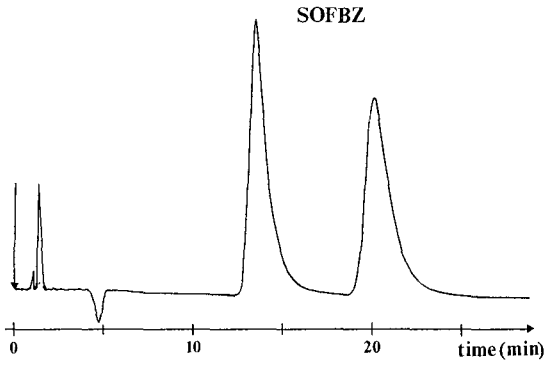

Fig. 3. Resolution of SOFBZ enantiomers on Chiral-AGP column. Reduced plate height, $h_{SOFBZ} \approx 25$. Mobile phase, 8 m*M* sodium phosphate buffer (pH 7.0); flow-rate, 0.9 ml min^{-1}; temperature, 25°C; UV detection at 220 nm.

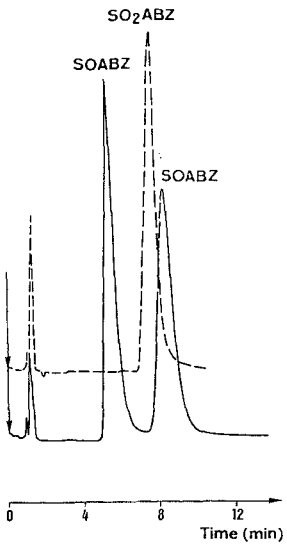

Fig. 4. Resolution of SOABZ enantiomers and comparison of elution of SO_2ABZ on Chiral-AGP column. Reduced plate height, $h_{SOABZ} \approx 40$. Mobile phase, 8 m*M* sodium phosphate buffer (pH 7.0); flow-rate, 0.9 ml min^{-1}; temperature, 25°C; UV detection at 220 nm.

undertaken to improve the resolution (especially in terms of efficiency) and prevent peak overlapping of SOABZ and SO_2ABZ. Other organic modifiers (various alcohols, aprotic polar solvents, etc.[11]) are under study, and also the influence of temperature.

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Note

High-performance liquid chromatographic separation of alkane-1,2-diol enantiomers on a chiral slurry-packed capillary column

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There is a trend to reduce the column diameter in high-performance liquid chromatography (HPLC), because capillary HPLC has some advantages over conventional HPLC using 4–5 mm I.D. columns, such as a potentially high column efficiency, low consumption of stationary and mobile phases, a small amount of sample and easy coupling with mass spectrometry[1–3]. Various organic and inorganic compounds have been analysed by reversed-phase, normal-phase and size-exclusion HPLC using columns of 1 mm or less I.D.[1–3]. Chiral-phase HPLC has also been carried out using 1 mm I.D. microbore columns[4–6]. However, capillary HPLC, in which the column I.D. is less than 1 mm, using chiral stationary phases has rarely been applied[7].

We have previously studied separations of lipid enantiomers and reported HPLC separations of enantiomeric monoacyl-[8], monoalkyl-[9] and diacylglycerols[10,11] as their 3,5-dinitrophenylurethane (3,5-DNPU) derivatives on chiral stationary phases. These separations were obtained using conventional stainless-steel columns of 4 mm I.D.

This paper describes the separation of enantiomers of alkane-1,2-diols as their bis(3,5-DNPU) derivatives by HPLC using a chiral slurry-packed capillary column. The separation of the enantiomers of lower homologues of 1,2-diols (C_2–C_8) using the conventional columns with a chiral stationary phase has been described recently by Pirkle *et al.*[12].

The higher alkane-1,2-diols are widely distributed as their diesters with long-chain fatty acids in mammalian skin lipids and bird waxes[13]. In this study, higher 1,2-diols were analysed using a chiral slurry-packed capillary column of 0.32 mm I.D. packed with particles containing the chiral stationary phase.

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EXPERIMENTAL

Samples

Alkane-1,2-diols were obtained from 2-hydroxy acid methyl esters by reduction with lithium aluminium hydride. (*RS*)-2-Hydroxyhexadecanoic acid, (*RS*)-2-hydroxyoctadecanoic acid and (*S*)- and (*RS*)-2-hydroxy-4-methylpentanoic acids (leucic acids) of purity >98% were purchased from Tokyo Kasei (Tokyo, Japan), Larodan Fine Chemicals (Malmö, Sweden) and Sigma (St. Louis, MO, U.S.A.), respectively, and converted to the methyl esters with diazomethane.

The bis(3,5-DNPU) derivatives were prepared from 1 mg of alkane-1,2-diols and about 2 mg of 3,5-dinitrophenyl isocyanate in toluene in the presence of pyridine[9]. The pure urethane derivatives were isolated from the reaction mixture by thin-layer chromatography (TLC) on silicic acid[9], using chloroform–acetone (24:1) as the developing solvent.

UV spectra of the bis(3,5-DNPU) derivatives were taken in ethanol on a Hitachi (Tokyo, Japan) U-2000 spectrophotometer.

HPLC

A Model LC-6A single-plunger pump (Shimadzu, Kyoto, Japan) was used for HPLC analysis and column packing. The chiral packing material, N-(*S*)-2-(4-chlorophenyl)isovaleroyl-D-phenylglycine chemically bonded to 5-μm particles of γ-aminopropylsilanized silica (Sumipax OA-2100; Sumitomo Chemical, Osaka, Japan), was slurry-packed into a fused-silica tube (40 cm × 0.32 mm I.D.) (QuadRex, New Haven, CT, U.S.A.) using carbon tetrachloride–liquid paraffin (1:1) as the slurry solvent[14]. The slurry reservoir (stainless-steel tubing, 2.2 mm I.D.) was connected directly to the fused-silica column through a reducing union. The slurry was added to the reservoir and brought into the capillary tube by the pump. The particles, tightly packed under high pressure, were retained in the column by a PTFE frit (Kusano Kagaku, Tokyo, Japan), which was supported by narrower fused-silica tubing (10 cm × 75 μm I.D.) connected to the detector. A Shimadzu SPD-6A variable-wavelength UV detector equipped with a laboratory-made flow cell (*ca.* 0.05 μl)[15] was set at 226 nm and 0.08 a.u.f.s. The detector cell was a 0.32 mm I.D. fused-silica tube, and the polyimide coating where the UV light beam crosses was removed by burning. The end of the slurry-packed capillary column was protected by a stainless-steel tube (2 cm × 1/16 in. O.D.), and was connected directly to a Rheodyne Model 7520 injector with a 0.2-μl sample chamber.

The bis(3,5-DNPU) derivatives dissolved in chloroform (HPLC grade) were injected with a 10-μl Hamilton syringe and analysed isocratically at ambient temperature (19–21°C) using *n*-hexane–1,2-dichloroethane–ethanol (all of HPLC grade) (40:12:3 or 20:5:1) as the mobile phase at a constant pressure of 10 kg/cm^2 (*ca.* 4 μl/min). Peak-area percentages and retention times were measured with a Shimadzu Chromatopac C-R6A integrator.

RESULTS AND DISCUSSION

Derivatives

As with monoacyl- and momoalkylglycerols[8,9], the two hydroxy groups of

alkane-1,2-diols reacted readily with 3,5-dinitrophenyl isocyanate in toluene solution in the presence of pyridine. The resulting bis(3,5-DNPU) derivatives were purified by preparative TLC on silicic acid.

In the TLC of the bis(3,5-DNPU) derivatives of monoalkylglycerols[6], *n*-hexane–1,2-dichloroethane–ethanol (20:5:1) was used as the developing solvent. This solvent system gave compact spots only when a small amount of sample (less than 1 mg) was applied on a TLC plate (20 cm × 20 cm, 0.25 mm thick layer). A similar observation was made for the bis(3,5-DNPU) derivatives of the alkane-1,2-diols. The solvent system employed in this study (chloroform–acetone, 24:1) gave clear chromatograms without tailing for the bis(3,5-DNPU) derivatives of both monoalkylglycerols and alkane-1,2-diols (R_F = 0.4 for hexadecane-1,2-diol), even when several milligrams of the samples were applied to the TLC plate. This is probably due to the greater solubility of the bis(3,5-DNPU) derivatives in this solvent.

Elemental analysis for the bis(3,5-DNPU) derivatives of octadecane-1,2-diol was as follows: found, C 55.10, H 6.57, N 11.58; calculated for $C_{32}H_{44}O_{12}N_6$, C 54.54, H 6.29, N 11.93%. The UV spectrum of the bis(3,5-DNPU) derivatives was essentially the same as that obtained for the monoacylglycerol bis(3,5-DNPU) derivatives (λ_{max} 226 nm)[8]. The 3,5-DNPU derivatives have sufficient sensitivity for HPLC detection over a wide range of UV wavelengths[8]. In our previous work[8–11], therefore, the 3,5-DNPU derivatives were detected at 254 nm, the wavelength generally used in UV detectors. To obtain a stable baseline at higher sensitivity, the bis(3,5-DNPU) derivatives in this study were monitored at 226 nm, which approximately doubles the sensitivity at 254 nm[8].

Separation

Fig. 1 shows the enantiomer separation of racemic hexadecane-1,2-diol as its bis(3,5-DNPU) derivatives on a chiral packed capillary column. Within 30 min, the racemate was clearly resolved into two components with nearly the same peak-area ratio, which demonstrates an effective separation into the enantiomers. The large and small peaks near 7 and 12 min were due to the solvent chloroform and the reagent isocyanate, respectively. A similar enantiomer separation to that in Fig. 1 was also obtained for racemic octadecane-1,2-diol. The chromatogram obtained for a mixture of the racemic and *S* enantiomeric 4-methylpentane-1,2-diol under the conditions used, showed clearly that the first and second peaks are the *R* and *S* enantiomers, respectively. Therefore, the enantiomer peaks obtained for racemic hexadecane- and octadecane-1,2-diols were also identified as shown in Fig. 1.

Table I gives chromatographic data for three racemic samples used. The enantiomer separations were examined using two mobile phases of different polarity. Although the lengthening of the retention times showed a slightly high separation factor and peak resolution, it also caused significant peak tailing. Such tailing is common for bands of high capacity factor (k'), and has been attributed to non-homogeneous adsorption sites[16]. The shifts to shorter retention times for the higher enantiomer homologues can be explained by the lower polarity of the alkane-1,2-diols with longer alkyl chains, which may interact weakly with the silica gel support. Similar shifts in the retention times of the enantiomer homologues were observed in the chiral-phase HPLC of the 3,5-DNPU derivatives of acyl and alkylglycerols with 4 mm I.D. columns containing the same packing material,

TABLE I

CHROMATOGRAPHIC DATA FOR ENANTIOMERIC ALKANE-1,2-DIOLS AS THEIR BIS(3,5-DNPU) DERIVATIVES ON A CHIRAL SLURRY-PACKED CAPILLARY COLUMN

RT = Retention time (min); k' = capacity factor; α = separation factor; R_s = peak resolution = $2(t_1 - t_2)/(w_1 - w_2)$, where t = retention time (min) and w = peak width (min); *TF* = tailing factor (%) = $100a/b$, where a and b are the baseline half-widths on the short- and long-time sides from the perpendicular drawn through the peak maximum, respectively.

Alkane-1,2-diol	*Enantiomer*	*Mobile phase (n-hexane–1,2-dichloroethane–ethanol)*									
		40:12:3					*20:5:1*				
		RT	k'	α	R_s	*TF*	*RT*	k'	α	R_s	*TF*
4-Methylpentane-	*R*	31.23	4.00	1.08	0.73	–	63.45	9.17	1.09	0.87	–
	S	33.27	4.33			–	68.73	10.01			–
Hexadecane-	*R*	21.69	2.44	1.24	1.87	96.2	41.48	5.65	1.27	2.03	85.2
	S	25.38	3.02			88.9	50.92	7.16			75.0
Octadecane-	*R*	20.84	2.34	1.25	1.86	94.8	40.83	5.54	1.28	2.00	86.1
	S	24.46	2.92			90.2	50.62	7.11			76.0

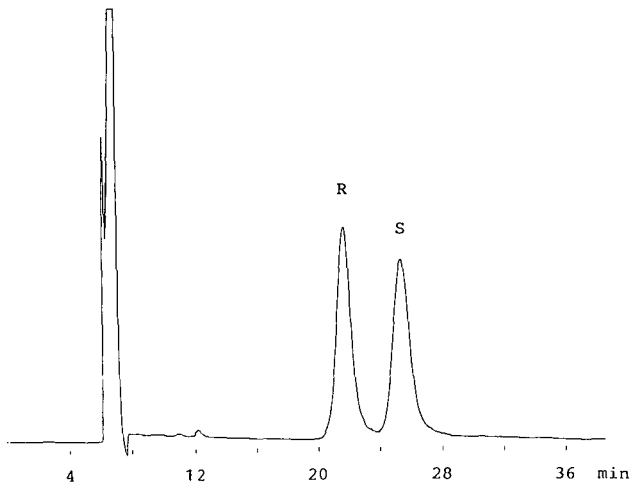

Fig. 1. Chiral-phase HPLC separation of hexadecane-1,2-diol enantiomers as their bis(3,5-DNPU) derivatives on a slurry-packed capillary column. Mobile phase: *n*-hexane–1,2-dichloroethane–ethanol (40:12:3). Other HPLC conditions as described in the text.

OA-2100[8–10]. Using the solvent system *n*-hexane–1,2-dichloroethane–ethanol (40:12:3), the separation factors of the *R* and *S* enantiomers of hexadecane- and octadecane-1,2-diols were 1.04 and 1.03, respectively, as calculated from the data in Table I. These values and the separation factors between *R* and *S* enantiomers hardly increased when using the lower polarity solvent system (20:5:1).

The chiral packed capillary column used in this study showed only 2800 theoretical plates for the *S* enantiomer peak of hexadecane-1,2-diol. The plate numbers were lower than that expected ($2 \cdot 10^4$–$3 \cdot 10^4$). This suggests that the column used in this study had not been well packed. Complete separations, however, were obtained for the long-chain alkane-1, 2-diol enantiomers as shown in Fig. 1 and Table I. The separation factor and peak resolution obtained for the *R* and *S* enantiomers of the long-chain alkane-1,2-diols are slightly higher than those of monoalkylglycerol enantiomers obtained using a 25 cm × 4 mm I.D. column packed with OA-2100[9], although the time of analysis was three times longer. The use of a column with more theoretical plates will give a higher resolution at a shorter retention time. The consumption of chiral material and mobile phase in this study were only *ca.* 1/100 and 1/250, respectively, of those in conventional HPLC (25 cm × 4 mm I.D. column, flow-rate 1 ml/min). The use of capillary columns therefore seems to be very economical in chiral-phase HPLC using expensive stationary phases.

The *S* enantiomers of monoacyl- and monoalkylglycerols as their bis(3,5-DNPU) derivatives were retained more strongly than the corresponding *R* enantiomers in conventional HPLC on the same chiral phase as that used in this study[8,9]. This supports the faster elution of the *R* enantiomers of alkane-1,2-diols as their bis(3,5-DNPU) derivatives on the chiral packed fused-silica column, and indicates that the mechanism of separation of alkane-1,2-diol enantiomers on the chiral stationary phase is essentially the same as that for monoacyl- and monoalkylglycerol

enantiomers. The formations of hydrogen bonds and charge-transfer complexes between the bis(3,5-DNPU) derivatives and the chiral stationary phase may contribute to the enantiomer separations, as discussed elsewhere[9,11].

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Note

Direct stereochemical resolution of enantiomeric amides via thin-layer chromatography on a covalently bonded chiral stationary phase

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In the past few years there has been a dramatic increase in the use of high-performance liquid chromatography (HPLC) chiral stationary phases (CSPs) for the direct stereochemical resolution of enantiomeric compounds. This technique has reached the point where it has been the subject of a number of recent reviews[1–3] and books[4,5]. The use of CSPs in gas–liquid chromatography has also grown and these advances have been discussed in a recent review[6]. The one chromatographic approach which has lagged in the application of CSPs is thin-layer chromatography (TLC).

Most of the initial work in enantioselective TLC using CSPs has centered on the development of phases for the stereochemical resolution of free and derivatized amino acids. The initial chiral resolution of an amino acid by a TLC-CSP was reported by Yuasa *et al.*[7] who resolved D,L-tryptophan on a TLC plate coated with microcrystalline cellulose. Another reported TLC-CSP utilized (+)-tartaric acid-impregnated silica gel plates which were used to resolve stereochemically phenylthiohydantoin amino acids[8]. Plates impregnated with (+)-ascorbic acid were also reported by these authors[8].

An alternative approach to the chiral resolution of free and derivatized amino acids has been chiral ligand-exchange chromatography[9–11]. In this approach reversed-phase TLC plates are impregnated with copper(II) complexed with an enantiomerically pure amino acid derivative. This method has also been used to determine the enantiomeric purity of commercial lots of L-3,4-dihydroxyphenylalanine (L-DOPA)[12] and D-penicillamine[13].

A TLC-CSP with a broader stereoselectivity has been reported by Armstrong and co-workers[14,15], who used β-cyclodextrin-bonded silica. This TLC-CSP has the capacity to resolve a variety of enantiomeric and diastereomeric compounds, including enantiomeric dansyl-amino acids, β-naphthylamide-amino acids and ferrocenyl compounds and the diastereomers quinine and quinidine[14]. In addition, the stereochemical resolutions obtained using the β-cyclodextrin TLC-CSP are comparable to those obtained using the same HPLC-CSP[15].

A class of HPLC-CSPs which also have a broad stereoselectivity are the

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Pirkle-type phases. A Pirkle-type TLC-CSP has been reported by Wainer *et al.*[16], who ionically bonded (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine (DNPG) to an aminopropyl-silica gel TLC plate. This support was able to stereochemically resolve racemic 2,2,2-trifluoro-1-(9-anthryl)ethanol with a stereochemical separation factor (α) of 1.50. This result was consistent with the α of 1.33 reported for the same enantiomers when resolved on the DNPG HPLC-CSP[17]. However, the TLC-CSP has a high UV background and the analytical applications of this support are currently limited.

An HPLC-CSP similar to DNPG has been described by Oi *et al.*[18], who reacted (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate with an aminopropyl HPLC support to produce a naphthylethylurea HPLC-CSP (Fig. 1). A variety of enantiomeric amines and acids were stereochemically resolved on this CSP as the 3,5-dinitrobenzoyl amide (DNB) or 3,5-dinitroanilide (DNAn) derivatives, including 1-phenylethylamine-DNB (α = 1.85), valine methyl ester-DNB (α = 2.02) and 1-methylphenylacetic acid-DNAn (α = 2.11).

In this study, we have reacted the isocyanate used by Oi *et al.*[18] with a commercially available aminopropyl HPTLC plate to form a naphthylethyl urea TLC-CSP. The resulting TLC-CSP was used to resolve stereochemically the same type of solutes as the naphthylethylurea HPLC-CSP and therefore, should have broad stereoselectivity. Although the napthylethyl chromophore of the present TLC-CSP also has a high UV absorptivity, experimentally we did not observe the detection problems that had been experienced with the DNPG plates. The DNB and DNAn derivatives used in this study could be detected by both short (254 nm) and long (360 nm) UV wavelengths with a limit of detection of 0.5 μg for one of the solutes. The naphthylethylurea TLC-CSP should provide useful alternative in enantioselective TLC.

EXPERIMENTAL

Materials

The (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate, nitroanilines, acid chlorides and (*R*)-(+)- and (*S*)-(−)-α-methylbenzylamines used in this study were purchased from Aldrich (Milwaukee, WI, U.S.A.). The (*R*)-(−)-ibuprofen was supplied by Upjohn (Kalamazoo, MI, U.S.A.) and the remaining solutes came from the stores of the U.S.

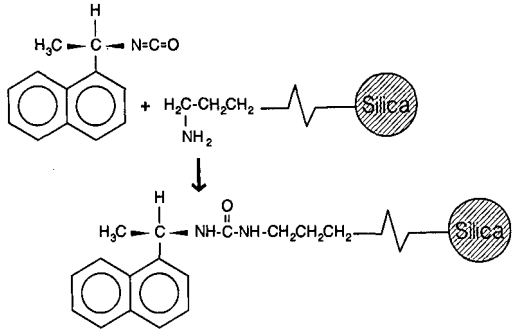


Fig. 1. The synthesis of the naphthylethylurea CSP.

Food & Drug Administration. The aminopropyl-silica gel TLC plates (NH_2, F-254s) were purchased from Alltech (Deerfield, IL, U.S.A.).

Preparation of TLC-CSP plates

A 1-g amount of the isocyanate was dissolved in 100 ml of methylene chloride. An aminopropyl HPTLC plate was soaked in 20 ml of the derivatization solution for 5 min. The plate was removed from the solution and air dried. The TLC-CSP plate was then washed by immersion in methylene chloride (twice) and air dried. The resulting plate was used without further treatment. The amount of isocyanate reagent bound to the plates was calculated from the loss of ultraviolet absorbance of the reagent solution, measured at 281 nm.

General procedure for the synthesis of amide derivatives

The amides derived from amines were synthesized by using the appropriate acid chloride according to a procedure outlined previously[19]. The amides derived from carboxylic acids were synthesized by a previously reported procedure which involved converting the acids to acid chlorides and condensing them with the appropriate nitroaniline[20].

Chromatographic conditions

The TLC-CSP plates were developed using a mobile phase composed of hexane–isopropanol–acetonitrile (20:8:1). The chromatography was carried out in saturated chromatographic tanks, except when the α-methylbenzylamine derivatives were studied.

Detection

The solutes were detected using both short (254 nm) and long (360 nm) UV wavelengths. The lower limit of detection for the 3,5-nitroanilide derivative of solute 1 was 0.5 μg.

Calculations

The R_F values for the solutes were calculated by averaging the results obtained from two separate experiments. The distribution ratios (D) and stereoselectivity values (α) were calculated using the R_F values according to the following equations:

$$D = R_F/(1 - R_F) \quad (1)$$

$$\alpha = D_2/D_1 \quad (2)$$

RESULTS AND DISCUSSION

Preparation of TLC-CSP plates

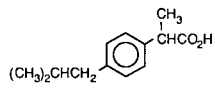
The TLC plates were prepared by facile room-temperature binding of (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate, dissolved in methylene chloride, to the aminopropyl-silanized silica support. The resulting naphthylethylurea chiral stationary phase is identical to the HPLC phase described by Oi *et al.*[18]. Under the conditions given in the Experimental section, the loading of the plates was 0.41 mmoles per gram of support, or about 86 mg isocyanate reagent per 10 × 10 cm plate.

Chromatographic results

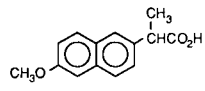
The structures of the compounds used in this study are presented in Fig. 2. Before chromatography on the TLC-CSP plates, the solutes were converted to amides.

A series of racemic α-methylarylacetic acids (solutes 1–5) was converted to the respective 3,5-dinitroanilides and chromatographed on the naphthylethylurea TLC-CSP (Table I). The enantiomeric DNAn derivatives of solute 1 had both the highest R_F values (0.45 and 0.28) and stereoselectivity ($\alpha = 2.10$) of the five solutes studied. The results for the solutes 2–5 were all similar. The R_F values of the least retained enantiomers of the enantiomeric pairs ranged from 0.24 to 0.30; the R_F values for the most retained isomers varied from 0.15 to 0.23. The stereoselectivity ranged from 1.65 to 1.79.

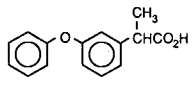
Two chiral amines, solutes 6 and 7, were derivatized with 3,5-dinitrobenzoyl chloride and the resulting amides chromatographed on the naphthylethylurea TLC-CSP (Table I). The R_F values for the enantiomeric DNB derivatives of solute 6 were 0.33 and 0.25 and the observed α was 1.48. For solute 7, the calculated R_F values were 0.37 and 0.31 with $\alpha = 1.31$.



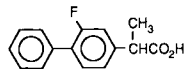
(1) Ibuprofen



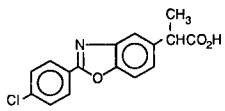
(2) Naproxen



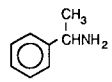
(3) Fenoprofen



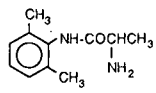
(4) Flurbiprofen



(5) Benoxaprofen



(6) α-Methylbenzylamine



(7) Tocainide

Fig. 2. Structures of the solutes used in this study.

TABLE I

CHROMATOGRAPHIC RESULTS

Compound (see Fig. 2)	*Derivative*[a]	R_F	α	*Order*
1	3,5 DNAn-	0.45, 0.28	2.10	*R,S*
2	3,5 DNAn-	0.24, 0.15	1.79	*R,S*
3	3,5 DNAn-	0.33, 0.23	1.65	ND[b]
4	3,5 DNAn-	0.33, 0.23	1.65	ND
5	3,5 DNAn-	0.30, 0.20	1.71	ND
6	3,5-DNB-	0.33, 0.25	1.48	*S,R*
7	3,5-DNB-	0.37, 0.31	1.31	ND

[a] 3,5-DNAn = 3,5-dinitroanilyl; 3,5-DNB = 3,5-dinitrobenzoyl.
[b] ND = not determined.

Stereoselectivity and solute structure

The relative chiral retention order of the enantiomers of solutes 1, 2 and 6 was determined by independent chromatography of at least one of the enantiomers of each pair. For solutes 1 and 2, the enantiomers with the (*S*)-configuration at the chiral center were more retained than the (*R*)-isomer while the opposite result was obtained for solute 6; *i.e.*, the (*R*)-enantiomer was more retained (Table I).

The inversion of the relative chiral retention order of an amide derived from an amine compared to that for an amide derived from a carboxylic acid has been observed on two HPLC-CSPs, the Pirkle-type DNPG-CSP[21] and the cellulose tribenzoate-CSP[22]. The results from these studies suggested that the position of the chiral center relative to the amide moiety is important due the interaction between the amide dipoles of the solute and CSP. This interaction not only plays a role in the formation of the diastereomeric solute–CSP complex but also orients the two molecules within this complex. This orientation ultimately determines the relative stability of the two diastereomeric complexes and, therefore, the relative retentions. The results from this study are consistent with these observations.

The effect of π-acidity on retention and stereoselectivity

The effect of the π-acidity of the aromatic portion of the amide moiety on R_F and α is presented in Table II. For the N-benzoyl derivatives of solute 6, R_F decreases with increasing π-acidity of the benzoyl moiety as represented by the Hammet substituent constants (σ)[23]. When the racemic 4-methylbenzoyl derivative ($\sigma = -0.17$) was chromatographed, the calculated R_F value was 0.59 while for the racemic 4-nitrobenzoyl derivative ($\sigma = +0.78$) the observed R_F value was 0.49. There was no observed chiral resolution. However, when the racemic 3,5-dinitrobenzoyl derivative was chromatographed, the calculated R_F values were 0.25 for the (*R*)-enantiomer and 0.33 for (*S*)-enantiomer with $\alpha = 1.48$.

A similar effect of π-acidity on retention and stereoselectivity was observed for three derivatives of solute 1 (Table II). When the racemic 3-nitroanilide and racemic 4-nitroanilide derivatives of solute 1 were chromatographed on the naphthylethylurea TLC-CSP, the calculated R_F values for the (*S*)-enantiomers were 0.53 and 0.51, respectively; the R_F values for both (*R*)-enantiomers were 0.59; the respective

TABLE II

EFFECT OF π-ACIDITY ON R_F AND α

σ = Hammet substituent constants[23].

Ar	σ	R_F	α
C_6H_5–CH(CH_3)–NH–CO–Ar			
4-CH_3-C_6H_4	−0.17	0.59	1.0
C_6H_5	0.00	0.56	1.0
4-NO_2-C_6H_4	+0.78	0.49	1.0
3,5-(NO_2)$_2$-C_6H_3	+1.42	0.33(*S*), 0.25(*R*)	1.48
4-(C_4H_9)–C_6H_4–CH(CH_3)–CO–NH–Ar			
3-NO_2-C_6H_4	+0.71	0.59(*R*), 0.53(*S*)	1.28
4-NO_2-C_6H_4	+0.78	0.59(*R*), 0.51(*S*)	1.38
3,5-(NO_2)$_2$-C_6H_3	+1.42	0.45(*R*), 0.28(*S*)	2.10

stereoselectivities were 1.28 and 1.38. The similarities in the chromatographic results between the two derivatives reflect the fact that the π-acidities of the 3-nitrobenzoyl ($\sigma = +0.71$) and 4-nitrobenzoyl ($\sigma = +0.78$) moieties are not significantly different. When the 3,5-nitrobenzoyl derivative ($\sigma = +1.41$) was chromatographed, there was a significant increase in the retention of both enantiomers, $R_F(S) = 0.28$ and $R_F(R) = 0.45$, and in the stereoselectivity, $\alpha = 2.10$.

These results suggest that one of the key aspects in the chiral recognition mechanism is a π–π interaction between a π-acidic moiety on the solute and the π-basic naphthyl moiety on the CSP. It also indicates that for the best chromatographic results, potential solutes for the naphthylethylurea TLC-CSP which contain an amine or a carboxylic acid moiety should be converted into the corresponding 3,5-dinitrobenzoyl amides or 3,5-dinitroanilides before chromatography.

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Note

Derivatization with 1,1-dimethylhydrazine for identification of carbonyl compounds resulting from ozonolysis

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Carbon–carbon double bonds are present in a variety of biologically important natural products. Biological activity is often directly related to the position of the double bond. A variety of methods are available for the location of double-bond positions[1–3]. Ozonolysis is one of the micro-methods which has been used extensively[4,5]. However, the carbonyl fragments arising from ozonolysis are often difficult to identify only by gas chromatographic (GC) retention times. Particularly, small carbonyl fragments, especially bifunctional carbonyls arising from polyunsaturated compounds, are difficult to identify. Moreover, the mass spectra of ozonolysis products are often featureless and rarely show observable molecular ions.

Derivatization with 1,1-dimethylhydrazine to form N,N-dimethylhydrazones has been used to characterize aldehydes by GC–mass spectrometry (GC–MS)[6]. In the present study, we describe the use of this derivatization reaction in the analysis of carbonyl products obtained from ozonolysis of unsaturated compounds.

EXPERIMENTAL

Materials

All chemicals including compounds identical to those found in some insect pheromones were purchased from Sigma (St. Louis, MO, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.) chemical companies. A mixture of (4*E*,6*Z*,10*Z*)-4,6,10-hexadecatrienyl acetate and (4*E*,6*E*,10*Z*)-4,6,10-hexadecatrienyl acetate was a gift of Dr. A. Cork (Overseas Development Natural Resources Institute, Kent, U.K.).

Apparatus and chromatographic conditions

Capillary GC was performed on a Hewlett-Packard 5890A instrument equipped with a flame ionization detector. A 25 m × 0.22 mm I.D. fused-silica column coated with methyl silicone gum OV-1 was used for analysis. The oven temperature was initially 60°C for 4 min then increased at a rate of 6°C/min to 280°C.

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GC–MS

The samples were analysed on a Hewlett-Packard 5890 gas chromatograph linked to a 5970B mass selective detector. A 20 m × 0.22 mm I.D. fused-silica capillary column coated with OV-101 methyl silicone was directly coupled to the detector. The temperature programme was identical to that used for GC. The detector was set to monitor masses *m/z* 35–350 and 70 eV EI spectra were recorded at about 1.5 scans/s.

Ozonolysis

Micro-ozonolysis was performed as described by Beroza and Bierl[4,5]. Each test substance (50 ng) in hexane (5 μl) was placed in the bottom of a cone-shaped micro-test tube and a slow stream of ozone (1–2 ml/min), generated by a micro-ozonizer (Supelco, Bellefonte, PA, U.S.A.), was passed for 1–2 min at room temperature. A solution of triphenylphosphane in hexane (1 μl, 1 mg/ml) was added to reduce the ozonides.

Derivatization with 1,1-dimethylhydrazine

1,1-Dimethylhydrazine (1 μl) was added to the ozonolysed solution in hexane. After 5 min at room temperature the solution was analysed by GC of GC–MS.

RESULTS AND DISCUSSION

The derivatization with 1,1-dimethylhydrazine proceeds smoothly under mild conditions. Aldehydes react essentially quantitatively and the reaction is complete in a few minutes. Presence of triphenylphosphane, used as a reducing agent to cleave ozonides, does not interfere with the reaction. The reaction mixture can be examined directly by GC or GC–MS.

The test compounds used were selected to represent different classes of compounds, with different degrees of unsaturation, encountered as pheromones. Table I summarizes the results of the present study. The mass spectra of N,N-dimethylhydrazones of aldehydes are very characteristic. A strong molecular ion is always present; therefore, it was possible to determine the molecular weights of the resulting aldehydes of ozonolysis readily. Even low-molecular-weight aldehydes, which are usually difficult to recognize by GC because of their high volatility, were identified without difficulty. Acetoxy aldehydes are particularly difficult to identify because authentic compounds are not usually available and their mass spectra seldom show molecular ions. All the N,N-dimethylhydrazone derivatives of acetoxy aldehydes identified in this study showed very prominent molecular ions (Table I).

In the case of (7*Z*,11*Z*)-7,11-hexadecadienyl acetate, we expected to see a peak for the mono- or di-hydrazone of succinic dialdehyde. However, we were unable to record a peak for this compound.

During our investigations, we found that it was possible to ozonolyse polyunsaturated compounds partially, one double bond at a time, by using a slow stream of ozone for a short time. The flow of ozone and duration of the reaction were controlled in such a manner that the polyunsaturated test compound peak did not disappear completely after ozonolysis. In fact, results obtained from a partial ozonolysis followed by derivatization with 1,1-dimethylhydrazine are more easily interpretable, and yield more information than those from a total ozonolysis. Fig. 1 shows the results of a partial ozonolysis of a mixture of (4*E*,6*Z*,10*Z*)-4,6,10-

TABLE I
N,N-DIMETHYLHYDRAZONES IDENTIFIED AFTER DERIVATIZATION OF OZONOLYSIS PRODUCTS

Ozonolysed compound	*Products identified*	*Mass spectral data, m/z (%)*
(7*Z*,11*Z*)-Hexadecadienyl acetate	$CH_3(CH_2)_3CH=N-N(CH_3)_2$	128(M^+,38), 113(8), 99(13), 86(59), 85(52), 71(13), 59(15), 44(100), 43(53)
	$CH_3CO_2(CH_2)_6CH=N-N(CH_3)_2$	214(M^+,34), 170(4), 155(5), 127(8), 113(10), 99(21), 86(100), 85(58), 59(37), 44(90), 43(82)
(*Z*)-5-Decenyl acetate	$CH_3CO_2(CH_2)_4CH=N-N(CH_3)_2$	186(M^+,17), 127(8), 111(6), 99(11), 86(18), 85(30), 59(20), 44(57), 43(100)
	$CH_3(CH_2)_3CH=N-N(CH_3)_2$	128(M^+,37), 113(6), 99(11), 86(56), 85(48), 71(12), 59(12), 44(100), 43(49), 42(46)
$C_6H_5CH=CHCH_2OCOCH_3$	$CH_3CO_2CH_2CH=N-N(CH_3)_2$	144(M^+,12), 102(3), 101(4), 85(12), 72(7), 57(8), 45(33), 44(37), 43(100), 42(63)
	$C_6H_5-CH=N-N(CH_3)_2$	148(M^+,100), 133(24), 118(27), 106(11), 104(13), 92(17), 90(22), 77(34), 63(8), 51(26), 44(35)
EZZ + EEZ[a]	$CH_3(CH_2)_4CH=N-N(CH_3)_2$	142(M^+,16), 127(4), 113(5), 99(8), 86(44), 85(37), 71(10), 59(24), 45(30), 44(100), 43(58), 42(52)
	$CH_3(CH_2)_4CH=CH(CH_2)_2CH=N-N(CH_3)_2$	196(M^+,6), 154(4), 86(7), 85(100), 44(40), 43(13), 42(21)
	$CH_3(CH_2)_4CH=CH(CH_2)_2CH=CH-CH=N-N(CH_3)_2$	222(M^+,8), 112(8), 111(100), 68(13), 44(28), 43(13), 42(18)
	$CH_3CO_2(CH_2)_3CH=N-N(CH_3)_2$	172(M^+,14), 112(9), 99(9), 97(28), 86(18), 85(19), 68(13), 45(19), 44(83), 43(100), 42(47)
	$CH_3CO_2(CH_2)_3CH=CH-CH=N-N(CH_3)_2$	198(M^+,16), 138(6), 123(12), 111(18), 95(15), 94(18), 82(12), 80(12), 68(13), 67(14), 59(18), 44(54), 43(100), 42(40)
	$CH_3CO_2(CH_2)_3CH=CH-CH=CH-(CH_2)_2CH=N-N(CH_3)_2$	252(M^+,9), 85(100), 44(32), 43(25), 42(15)

[a] (4*E*,6*Z*,10*Z*)-4,6,10-hexadecatrienyl acetate and (4*E*,6*E*,10*Z*)-4,6,10-hexadecatrienyl acetate.

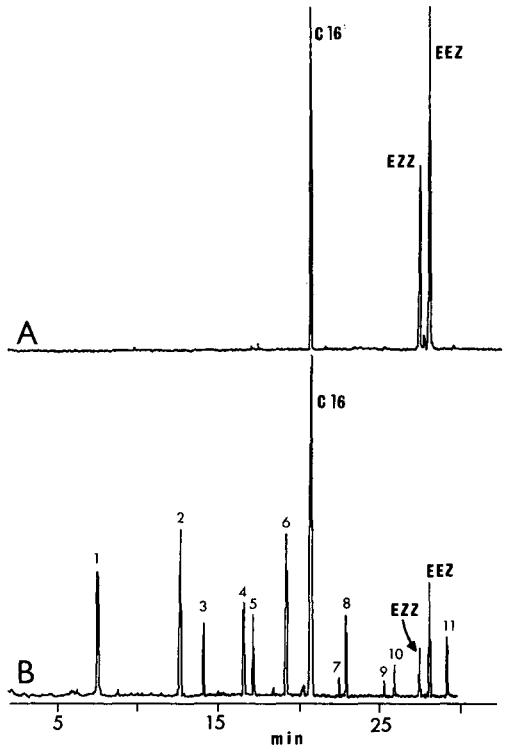

Fig. 1. Reconstructed ion chromatograms obtained on a methylsilicone column (20 m × 0.22 mm I.D., 4 min at 60°C, 6°C/min to 280°C, splitless injection). (A) (4*E*,6*Z*,10*Z*)-4,6,10-hexadecatrienyl acetate [EZZ] and (4*E*,6*E*,10*Z*)-4,6,10-hexadecatrienyl acetate [EEZ] together with hexadecane [C16] as an internal standard. (B) After partial ozonolysis, followed by derivatization with 1,1-dimethylhydrazine. Peaks 1–10 are 1,1-dimethylhydrazones of the following: 1 = hexanal; 2 = 4-acetoxybutanal: 3 = unidentified; 4 = unidentified; 5 = (*Z*)-4-decenal; 6 = (*E*)-6-acetoxy-2-hexenal; 7 = (2*Z*,6*Z*)-2,6-dodecadienal; 8 = (2*E*,6*Z*)-2,6-dodecadienal; 9 = (4*E*,6*Z*)-10-acetoxy-4,6-decadienal; 10 = (4*E*,6*E*)-10-acetoxy-4,6-decadienal. Peak 11 is triphenylphosphane.

hexadecatrienyl acetate and (4*E*,6*E*,10*Z*)-4,6,10-hexadecatrienyl acetate. The latter is the major component of the sex pheromone of cocoa pod borer moth, *Conopomorpha cramerella*[7]. If partial ozonolysis breaks only one double bond at a time, six aldehyde fragments are expected from each geometric isomer of 4,6,10-hexadecatrienyl acetate. The masses of expected carbonyl compounds after derivatization with 1,1-dimethylhydrazine is illustrated in Fig. 2. All the expected carbonyl compounds were detected

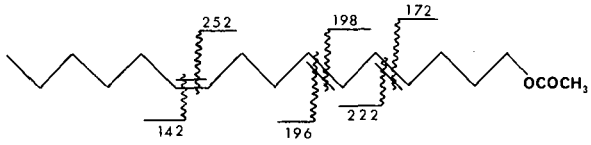

Fig. 2. Molecular masses of N,N-dimethylhydrazones expected from partial ozonolysis fragments of (4*E*,6*E*,10*Z*)-4,6,10-hexadecatrienyl acetate.

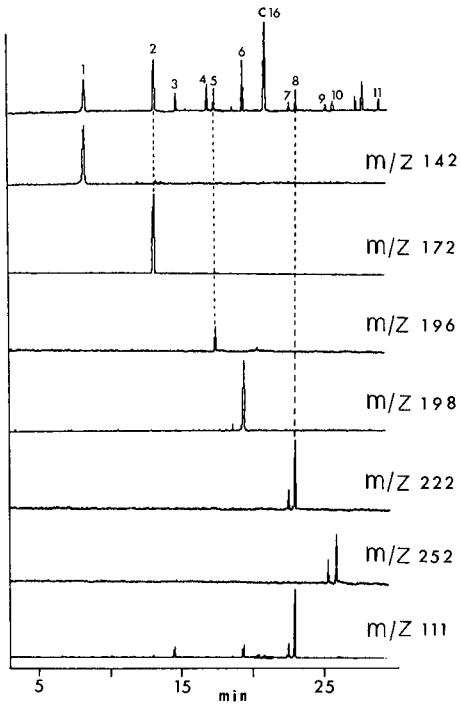

Fig. 3. A reconstructed gas chromatogram and seven mass chromatograms for the ions *m/z* 142, 172, 196, 198, 222, 252, and 111, obtained from GC–MS analysis of derivatized partial ozonolysis products of (4*E*,6*Z*,10*Z*)-4,6,10-hexadecatrienyl acetate and (4*E*,6*E*,10*Z*)-4,6,10-hexadecatrienyl acetate. Refer to Fig. 1 for peak assignments and chromatographic conditions.

in the ozonolyzed mixture (Fig. 1). The peaks 1, 2, 5 and 6 originated from both (*E*,*Z*,*Z*)- and (*E*,*E*,*Z*)-isomers, whereas paks 7 and 9 were fragments of the (*E*,*Z*,*Z*)-isomer, and peaks 8 and 10 were those from (*E*,*E*,*Z*)-isomer.

Although 50 ng of each polyunsaturated compound was used customarily in the present study, MS analysis (Fig. 3) and single-ion monitoring investigations revealed that only a few nanograms of the starting material was sufficient for a complete analysis. Peaks 3 [*m/z* 44(100), 60(80), 85(64), 111(19)] and 4 [*m/z* 44(100), 83(17), 86(27), 125(50)] were two unidentified products. They appeared in all chromatograms after derivatization, independent of which compound was ozonolysed.

N,N-Dimethylhydrazone spectra are not only useful for mass determination of aldehyde fragments; they also yield structural information. The hydrazones of saturated aldehydes show a significant fragment ion at *m/z* 86 resulting from a McLafferty rearrangement[8]. This ion is also seen in the spectra of unsaturated aldehydes if the first double bond is not in the vicinity of the carbonyl group[6]. α,β-Unsaturated aldehydes are unable to yield this McLafferty ion; they undergo an allylic fission to give a significant ion at *m/z* 111 due to the ion $CH_2=CH-CH=CH-N=N^+(CH_3)_2$ (ref. 9). MS search for the ion *m/z* 111 indicated the peaks 6, 7, and 8 are α,β-unsaturated aldehydes (Fig. 3). A McLafferty ion is also not possible when a double bond is present at C-4. Such unsaturated aldehydes give

a very significant ion at m/z 85 due to the ion $CH_2=CH-N=N^+(CH_3)_2$ (ref. 8). In fact this ion is the base peak of the spectra of the GC peaks 5, 9 and 10 of Fig. 3.

The partial ozonolysis procedure appears to offer great potential in the structure elucidation of polyunsaturated insect pheromones[3]. It is currently being applied in our laboratory for the structure elucidation of a number of unknown polyunsaturated pheromones. The results will be reported elsewhere.

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The authors thank Dr. Alan Cork for a kind gift of (4*E*,6*Z*,10*Z*)-4,6,10-hexadecatrienyl acetate and (4*E*,6*E*,10*Z*)-4,6,10-hexadecatrienyl acetate, and Dr. O. Vostrowsky for critically reading the manuscript.

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Note

Reversed-phase liquid chromatography for enrichment of very-long-chain fatty acids and their identification by gas chromatography–mass spectrometry

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Very-long-chain fatty acids (VLCFA) with more than 23 carbon atoms occur more rarely[1] in nature than those with the usual 14–24 carbon atoms in the molecule. They are found in both the plant and animal kingdoms. Two basic types of VLCFA can be distinguished, the saturated fatty acids (FA), with accompanying monounsaturated FA and polyenoic VLCFA. With the exception of, *e.g.*, plant wax, VLCFA are present at trace levels not exceeding 1–2% of the total FA in plants and animals and also in microorganisms. Palmitic and oleic acid represent major acids occurring in living organisms.

For the detection of trace amounts of VLCFA, several methods have been developed. Gas chromatography (GC) has often been used for qualitative and quantitative determinations, but not for the enrichment of a sample with VLCFA. Preparative GC was applied to the isolation of fatty acid methyl esters (FAME) with short and medium-length chains up to C_{18} for about 20 years. The oldest method is based on the interesting phenomenon of the solubility of sodium salts of VLCFA in non-polar solvents, *e.g.*, light petroleum[2]. Other methods are based on chromatographic methods, mainly with separation in the reversed-phase mode[3–5]. The principle of this method, employed by Takayama *et al.*[3], consists in a partial separation of FA according to the chain length on Sephadex LH-20. Two partially separated maxima were thus obtained; the first eluted contained VLCFA in the range C_{35}–C_{55} and the second C_{24}–C_{35} VLCFA.

Reversed-phase high-performance liquid chromatography (RP-HPLC) has been used by many workers, but most of them have employed this method for the separation of the individual FA in either the analytical[6–9] or preparative mode[10,11]. On the basis of our previous experience with semi-preparative RP-HPLC[12,13], we

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have used this method for the rapid enrichment of the VLCFA fraction with minimal losses. To demonstrate the suitability of the method used, FA mixtures containing VLCFA prepared from the fungi of the Basidiomycetes family[14] or rat Harderian gland were utilized. In these samples, the presence of VLCFA had been demonstrated by gas chromatography–mass spectrometry.

EXPERIMENTAL

All solvents were distilled and oxygen was removed before use. Acid standards (18:0, 20:0, 24:0 and 28:0) were purchased from Sigma (St. Louis, MO, U.S.A.). The natural mixture was composed of the methyl esters of the acids that we had isolated from the fungus *Ganoderma applanatum* (Basidiomycetes)[14] and from rat Harderian glands[15].

Separation of VLCFA in the form of salts

A standard mixture of VLCFA (6 mg) composed of the equal masses of acids (18:0, 24:0 and 28:0) was dissolved in 1 ml of a 0.1 *M* solution of a corresponding hydroxide (sodium, potassium, ammonium, calcium) and extracted three times with 1 ml of hexane. The combined extracts were dried by evaporation and converted into methyl esters by using 14% boron trifluoride in methanol.

Thin-layer chromatography (TLC)

A 1-mg amount of a standard mixture (or of the natural mixture) in the form of FAME was placed on TLC plates (silica gel $100F_{254}$, precoated layer thickness 0.25 mm, 20 × 20 cm; Merck, Darmstadt, F.R.G.) and chromatographed by using chloroform–benzene (1:1). The compounds were detected at 254 nm; the corresponding spots were scraped off and eluted with diethyl ether.

Reversed-phase TLC (RP-TLC)

A 1-mg amount of a standard FAME mixture was placed on RP-TLC plates (RP-18 F_{254s}, layer thickness 0.25 mm, 20 × 20 cm; Merck) and chromatographed with acetic acid–acetonitrile–tetrahydrofuran (30:30:40). After the chromatogram had been developed, the spots detected at 254 nm were scraped off and eluted with diethyl ether (R_F 0.52 = FAME 18:0, R_F 0.35 = FAME 24:0, R_F 0.24 = FAME 28:0). With the natural mixtures, a band in the range R_F = 0.40–0.05 was collected by scraping.

Reversed-phase high-performance liquid chromatography

A semi-preparative separation of FAME was carried out by using a Varian 8500 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.). A stainless-steel column (250 mm × 8 mm I.D.; Separon SGX C_{18} sorbent, 7 μm) (Laboratorní přístroje, Prague, Czechoslovakia) was employed. The flow-rate of the mobile phase was 2.0 ml/min. In the time interval 0–60 min the column was eluted with methanol that was later replaced with diethyl ether (15 min). The column was again conditioned with methanol (15 min). The fraction collected up to 17 min was discarded, and that obtained within the interval 17–90 min was used for further analysis (detection at 208 nm).

Gas chromatography

A Sigma 3B apparatus (Perkin-Elmer, Norwalk, CT, U.S.A.) with a fused-silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) having a chemically bonded SPB-5 stationary phase (Supelco, Gland, Switzerland) was employed. The samples were chromatographed under the following conditions: temperature programme, 230–320°C at 3°C/min; temperature of the injector (splitless) and detector (FID), 340°C; carrier gas, hydrogen at a linear flow-rate of 40 cm/s.

Gas chromatography–mass spectrometry

The mixture of FAME was analysed on a Shimadzu Model QP 1000 quadrupole GC–MS system (Shimadzu, Tokyo, Japan) using an SPB-1 fused-silica capillary column (Supelco, Bellefonte, PA, U.S.A.) (60 m × 0.25 mm I.D.) with a film thickness of 0.25 μm. The injector temperature (splitting ratio 1:20) was 280°C and the oven temperature was increased from 160 to 330°C at 6°C/min.

RESULTS AND DISCUSSION

Four methods as described below were used for the enrichment with VLCFA. Although a loss of VLCFA has been described in the literature[2], we were not able to enrich selectively the light petroleum extract with VLCFA in this way. By using the above-mentioned salts, none of the extractions carried out resulted in any enrichment with 24:0 and/or 28:0. For example, by using sodium salts of the model mixture, 3.1 μg of FA were obtained in three extractions, the proportions of 18:0, 24:0 and 28:0 being 1.00:1.01:1.01, which, from the practical point of view, represented a negligible enrichment of the hexane fraction with VLCFA.

Another method that did not yield the expected results was preparative TLC on silica gel. Again, procedures had been described[16], including the composition of the mobile phase and the sorbent activity, ensuring the separation of homologues exhibiting differences of four and more CH_2 groups. In this case, the separation of 18:0 from 24:0 was good but the separation of 24:0 from 28:0 was poor. However, normal-phase TLC could not be adapted for the chromatographic separation of these complex mixtures.

Reversed-phase chromatographic methods, either RP-TLC or, better, RP-HPLC, were found to be successful. When RP-TLC was used, the proportions in the model mixture were changed from 1:1:1 (18:0, 24:0, 28:0) to 1:10:13, which represented an enrichment with VLCFA by one order of magnitude.

Even though a separation of the individual FAME has been reported in the literature[17,18], we were not able to separate the individual homologues in the natural mixture. Another disadvantage that we also were not able to overcome, in spite of the use of an antioxidant (*tert.*-butylhydroxyanisole, BHA) in the mobile phase, saturation of the solvents with nitrogen and subsequent separation of the compounds in a nitrogen atmosphere was the oxidative degradation of the unsaturated bonds. The loss of 24:1 can be caused by a process other than by peroxidation, *e.g.*, by smearing of the zone during TLC. As for the detection of peroxide(s), they are thought to have a significantly higher molecular weight (by at least two oxygen atoms) and, therefore, exhibit longer elution times and are thermally degradable at the elution temperature. This is why it would be necessary to carry out the derivatization, which would,

however, result in another increase in molecular weight and in the elution temperature and time, and hence the compounds would not be eluted from the column as one peak within a reasonable time. The proportion of 24:1 to 24:0 in the original mixture was 0.87[14], whereas after RP-TLC it was as low as 0.23, which unequivocally indicated a loss of 24:1.

The best results were obtained by using RP-HPLC. Methyl arachidate was chosen as the standard, having a retention time of 17 min under the conditions used. Detection at 208 nm was very difficult, especially during the preparation, and, therefore, the conditions developed for the analytical mode were employed. The methyl ester 20:0 was chosen because major acids up to C_{20} were present in the two natural mixtures. The separation efficiency is readily seen in Fig. 1, where a chromatogram of the original mixture obtained from *Ganoderma applanatum* (a) is shown together with the fraction acquired by RP-HPLC (b), whose retention time was longer than 17 min.

Unfortunately, no UV-absorbing derivative of such long FA can be chromatographed by GC. As far as the commonest, UV-absorbing, compounds are concerned, *viz.*, bromophenacyl esters, their polarity and molecular weight increase so much that during the injection and mainly during a GC run they are degraded and cannot be eluted from the column.

The disadvantage met during RP-TLC, *viz.*, the oxidative degradation of monoenoic FAME, was minimized by using RP-HPLC (the proportion of 24:1 to 24:0 was 0.87 in the original mixture and changed only slightly to 0.84 after RP-HPLC). As a result of the enrichment of the individual fractions with VLCFA, so far unknown homologues were identified (Fig. 1b). A mass spectrum of the highest homologue found (34:0 FAME) is shown in Fig. 2, indicating the presence of these acids in the sample.

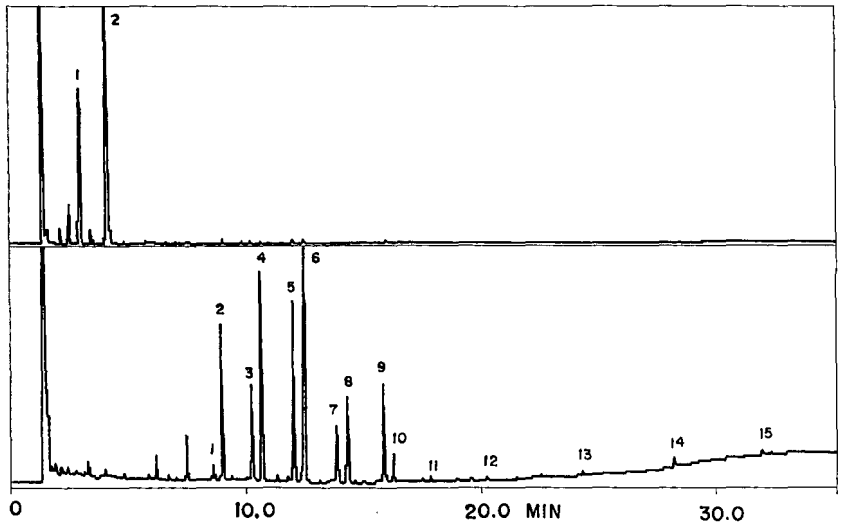

Fig. 1. GC of FAME of fungus *Ganoderma applanatum*. (a) (upper trace), total FAME. Peaks: 1 = 16:0; 2 = 18:1. (b) (lower trace), FAME after preparative RP-HPLC. Peaks:1 = 22:1; 2 = 22:0; 3 = 23:1; 4 = 23:0; 5 = 24:1; 6 = 24:0; 7 = 25:1; 8 = 25:0; 9 = 26:1; 10 = 26:0; 11 = 27:0; 12 = 28:0; 13 = 30:0; 14 = 32:0; 15 = 34:0. Only major FAME are shown.

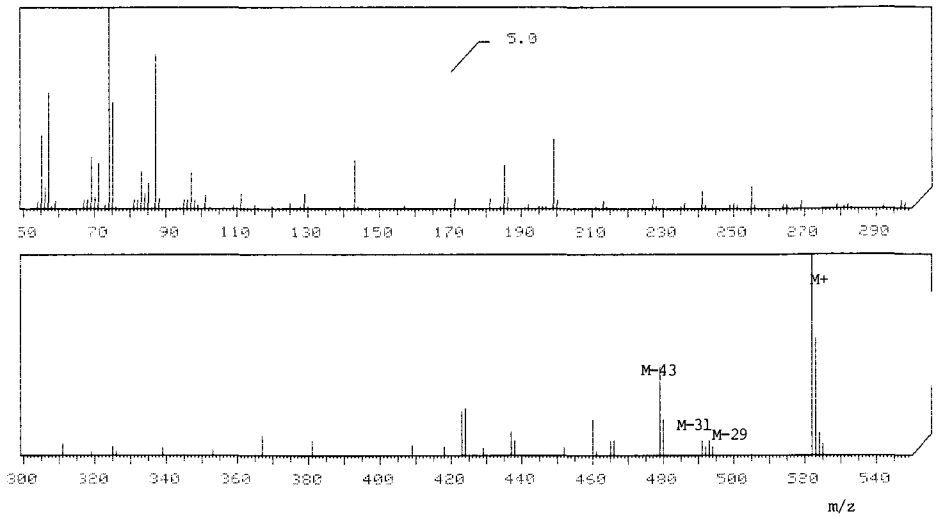

Fig. 2. Electron impact mass spectrum of 34:0 FAME [see peak 15 in Fig. 1b (lower)]. M^+ = molecular ion.

For the saturated FAME, the significant M^+ ions are $M-29$, $M-31$ and $M-43$, which were ubiquitous (see Fig. 2 for FAME 34:0); for the monoenic acids, M^+ together with significant ions $M-32$ and $M-74$ were present. All FAME show the presence of the ions of the general type, *viz.*, $(CH_2)_nCOOCH_3$, where n = 2,6,10,14,..., which corresponds to m/z values of 87, 143, 199 and 255, respectively. These ions (n = 2, m/z 87; n = 6, m/z 143; n = 10, m/z 199; n = 14, m/z 255; n = 18, m/z 311; n = 22, m/z 367; n = 26, m/z 423; n = 30, m/z 479) were also present in FAME 34:0 (see Fig. 2). It is concluded that the only suitable method for the enrichment of the samples with VLCFA is RP-HPLC. This method provides excellent yields and no degration of FAME takes place, in contrast to RP-TLC. The latter method therefore seems to be suitable only for saturated FAME.

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Note

High-performance liquid chromatography of chlorophylls and carotenoids from vegetables

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The photosynthetic pigments, chlorophylls and carotenoids, belong to the group of isoprenoid plant lipids called prenyl lipids. Several procedures have been developed for the separation of these pigments, including paper (PC), column (CC), thin-layer (TLC) and more recently high-performance liquid chromatography (HPLC)[1]. The simultaneous separation and detection of chlorophylls and carotenoids have been achieved by HPLC with stepwise gradient elution systems on reversed-phase columns[2–7]. These systems, however, were either time consuming or very expensive. The isocratic separation of prenyl pigments by adsorption and reversed-phase HPLC have also been reported[8]. Apart from polar xanthophylls and β-carotene, only chlorophyll *a* and *b* could be separated. The chromatographic determination of chlorophyll degradation products required a second elution step with a stronger solvent mixture on another stationary phase.

There are many possibilities for increasing the efficiency of HPLC techniques for the separation of photosynthetic pigments under isocratic conditions, *e.g.* the use of a suitable chromatographic adsorbent and a mobile phase that increases the separability of a wide variety of pigments. The objective of this work was to devise an HPLC method for the simultaneous separation of chlorophylls and carotenoids and their derivatives on a C_{18} column under isocratic conditions.

EXPERIMENTAL

Preparation of pigments

Pigments of green vegetables were extracted with methanol and carbon tetrachloride as described previously[9]. Chlorophylls and pheophytins were prepared from raw, blanched and cooked spinach leaf extracts by TLC on cellulose sheets (MN-300; Merck, Darmstadt, F.R.G.) developed with two solvent systems[10].

Saponification of chlorophylls and pheophytins to chlorophyllids and pheophorbides was carried out by refluxing the extracted pigments for 10 min with 30 ml of 0.5% methanolic potassium hydroxide solution at the boiling point of methanol in the presence of ascorbic acid. It should be noted that saponification of chlorophylls by a concentrated alkaline solution leads to the formation of highly polar pigments that cannot be recovered by water-immiscible solvents. The saponified pigments were

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then extracted from methanol by shaking gently with benzene or chloroform. Following removal of the organic solvent under vacuum, the pigments were separated on a cellulose layer, developed with *n*-heptane–pyridine (7:3, v/v)[10].

Oxygen-containing carotenoids were separated and prepared on cellulose layers eluted with different mobile phases[11].

Identification of pigments

Chlorophyll and carotenoid-type pigments and their derivatives thus prepared were identified according to their R_F values obtained from TLC analysis and their visible absorption characteristics compared with those in the literature[4,6,12]. The identified pigments were scraped off the TLC plate and eluted with suitable solvents. After evaporating the solvent, the residues were dissolved in the minimum volume of the HPLC eluent and applied to the column for retention time measurement and to scan their absorption maxima between 200 and 700 nm (Table I). β-Carotene was identified and quantified by using the standard pigment (Sigma, St. Louis, MO, U.S.A.). Chlorophyllides and pheophorbides were further identified by comparison with authentic standards prepared by a method that included chlorophyllase-[13] and acetic acid-catalysed[14] reactions. The tentative identification of the oxidation products of chlorophylls (peaks X and Y in Table I) was achieved by special experiments

TABLE I

SPECTRA AND CHROMATOGRAPHIC PROPERTIES OF PIGMENTS ISOLATED FROM GREEN VEGI TABLES AND SEPARATED BY HPLC

Eluent, acetonitrile–methanol–ethyl acetate (53:40:7); flow-rate, increased from 0.5 to 2 ml/min at the 22nd min c elution.

Peak No.	*Pigment*	$\lambda_{max.}$ *(nm)*	*Retention time (min)*	*Capacity factor (k')*	*Peak area* $(mm^2)^a$	*C.V.* $(\%)^b$
1	Chlorophyllid *b*	433, 660	4.27	0.58	2.2	1.6
2	Chlorophyllid *a*	408, 675	4.70	0.75	17.5	1.4
3	Neoxanthin	417, 443, 472	5.61	1.11	23.4	0.8
4	Violaxanthin	422, 447, 475	6.38	1.38	52.5	0.7
5	Lutein epoxide	417, 446, 477	7.15	1.66	16.3	1.2
6	Lutein	426, 453, 478	8.50	2.16	220.4	1.0
7	Neolutein	415, 439, 468	9.07	2.37	43.5	0.8
8	Chlorophyll *b*	462, 660	14.26	4.30	415.6	0.6
9	Chlorophyll *b'*	460, 660	14.86	4.50	46.7	1.2
10	Chlorophyll *a*	432, 668	20.61	6.51	1170.2	1.8
11	Chlorophyll *a'*	428, 667	22.30	7.28	230.8	2.3
12	Pheophytin *b*	436, 659	25.90	8.62	34.6	0.9
13	Pheophytin *a*	415, 669	29.05	9.70	9.2	1.5
14	β-Carotene	456, 477	30.38	10.29	96.4	0.4
15	Pyropheophytin *a*	414, 668	33.67	11.52	242.3	0.7
16	Unidentified	415, 669	34.75	13.91	46.8	0.9
X	Chlorophyll *b* oxidation product	460, 663	12.73	3.73	45.2	1.7
Y	Chlorophyll *a* oxidation product	430, 669	16.96	5.30	60.4	1.9

[a] Detection was carried out at 430 nm for carotenoids and 660 nm for chlorophylls.

[b] Coefficient of variation for six runs of the same sample.

including incubation of the extracted pigments with soybean lipoxygenase and linoleic acid as described previously[15].

High-performance liquid chromatography

The extracts (20 μl) were injected into a Beckman liquid chromatograph equipped with a Model 114 M solvent-delivery module pump, a Model 420 controller, a Model 340 organizer and a Model 165 variable-wavelength detector. The detector signals were electronically recorded with a Shimadzu Type GR3A integrator.

Separations were performed on a Chromsil C_{18} (10 μm spherical particles) stainless-steel column (25 cm × 4.6 mm I.D.) (Labor-MIM, Estergom, Hungary). The mobile phase was acetonitrile–methanol–ethyl acetate (53:40:7). The flow-rate was 0.75 ml/min at the beginning of run and then elevated to 2 ml/min as shown on the figures. Detection was carried out at different wavelengths.

Reagents

Acetonitrile, methanol and ethyl acetate were of HPLC grade (Reanal, Budapest, Hungary). They were used after redistillation, filtration and degassing. Benzene and chloroform were of technical grade and were used without purification.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of a methanol–carbon tetrachloride extract obtained from raw green pea. The mobile phase was acetonitrile–methanol–water (50:45:5) and the pigments were eluted at a flow-rate of 1 ml/min. This elution system gave a good separation of the polar components, which eluted first on a reversed-phase column, but failed to resolve the pigments of low polarity such as β-carotene and pheophytins, with retention times of less than 50 min. The appearance of broad peaks for β-carotene and pheophytins made their detection and determination difficult and unsatisfactory under the conditions used.

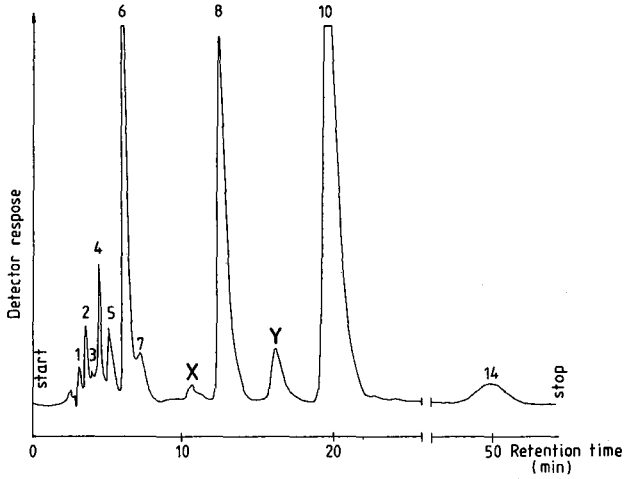

Fig. 1. HPLC profile of green pea extract on a Chromsil C_{18} column eluted with acetonitrile–methanol–water (50:45:5) at a flow-rate of 1 ml/min. Detection at 438 nm.

Addition of water to the elution mixture greatly extends the elution time[2]. Therefore, we attempted to improve the resolution of the apolar pigments by replacing water with ethyl acetate. This modification resulted in a satisfactory separation of most of the chlorophylls and carotenoids with symmetrical peaks, as shown in Fig. 2. Similar resolution profiles of some vegetable extracts have been obtained with gradient elution systems that take a long time for a complete run[2,4].

Another modification was the programmed increase in flow-rate, which shortened the retention time of the apolar pigments and increased their detectability even when they were present at relatively low concentrations.

Chlorophylls and their derivatives could be distinguished from other pigments by detecting the separated components at 650 nm (Fig. 3). Unesterified derivatives of chlorophylls such as chlorophyllides eluted first. The ratio of chlorophyllid *a* to *b* differs in the different vegetables. One-step separation and detection of such metabolites opens up a new possibility for using HPLC in the rapid analysis of enzyme- and non-enzyme-catalysed hydrolysis of chlorophylls to phytyl alcohol and unesterified derivatives.

As the coefficients of variation (C.V.) between runs for each chlorophyll- and carotenoid-type pigment did not exceed 2%, the method can be considered to be precise and accurate for the analysis of a wide variety of components (Table I). The relatively high C.V. for some xanthophylls, chlorophylls *a* and oxidation products indicate their susceptibility to degradation by oxidation and isomerization processes.

Detection of the separated pigments at different wavelengths indicated that 415–420 nm was the best range for the simultaneous detection of xanthophylls, carotenes, chlorophylls and pheophytins. The most familiar derivatives of chlorophylls

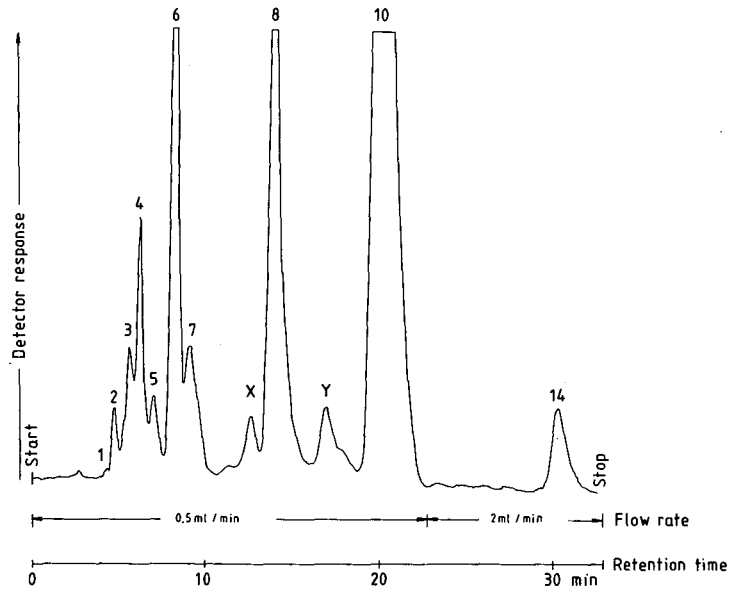

Fig. 2. HPLC profile of celery leaf extract on a Chromsil C_{18} column eluted with acetonitrile–methanol–ethyl acetate (53:40:7) with increase in flow-rate. Detection at 438 nm.

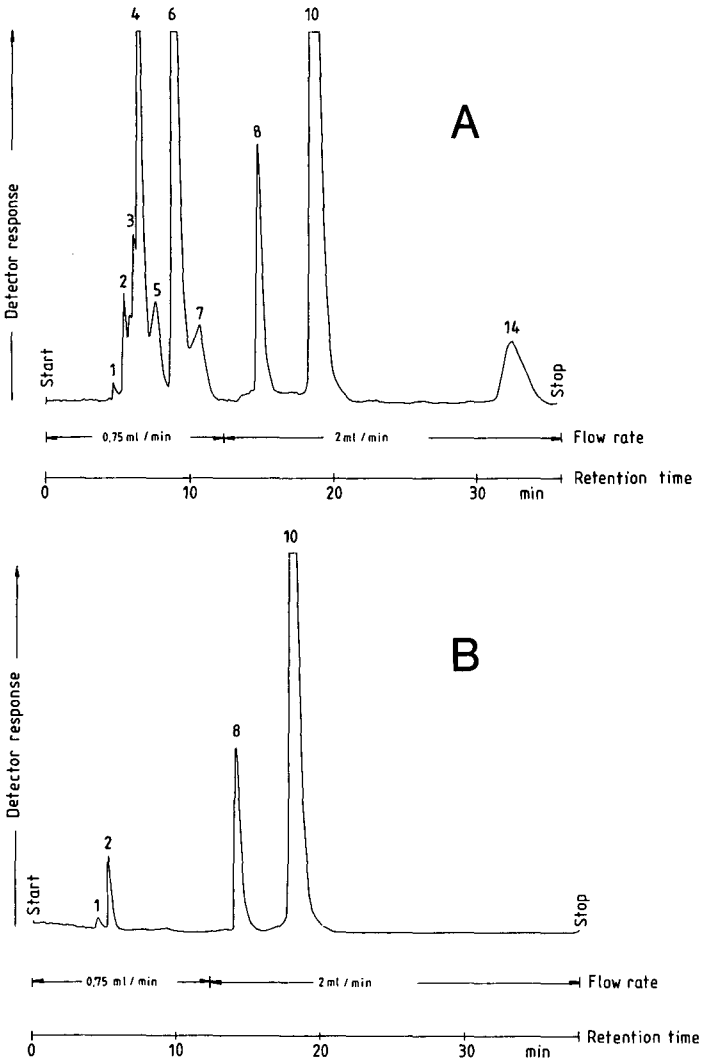

Fig. 3. HPLC profiles of freshly harvested spinach leaf extract on a Chromsil C_{18} column eluted with acetonitrile–methanol–ethyl acetate (53:40:7). Detection: (A) at 438 nm; (B) at 650 nm.

are pheophytins and pyropheophytins *a*. Their detection in the above range could be achieved even when they were present at relatively low concentrations. Shorter detection wavelengths have been reported for the chlorophylls, pheophytins and pheophorbides *a*[6,16].

Fig. 4 shows the chromatograms of chlorophylls and carotenoids in raw and blanched celery leaves. Bleaching for 2 min at 100°C resulted in the appearance of chlorophyll *a'* and *b'*, the C-10 epimers of chlorophyll *a* and *b*, respectively. These isomers had higher retention values than their parent pigments. Such HPLC behaviour has been observed for the same compounds extracted from raw blanched spinach[17].

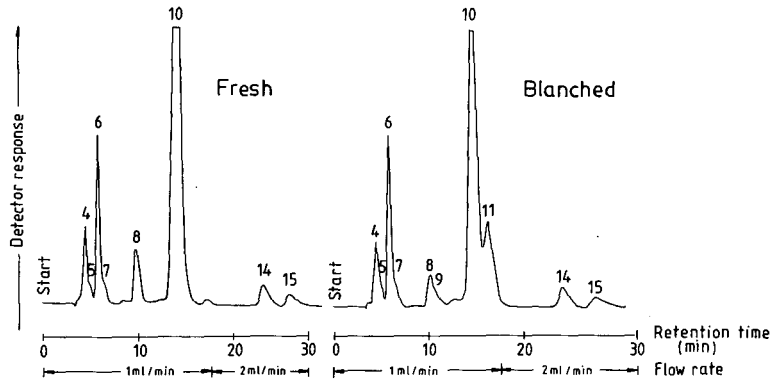

Fig. 4. Separation of chlorophylls and carotenoids of fresh and blanched celery leaves on a Chromsil C_{18} column eluted with acetonitrile–methanol–ethyl acetate (53:40:7). Detection at 415 nm.

Cooking of vegetables with brine or an acidic solution leads tot the formation of pheophytins from chlorophylls. As chlorophyll *a* is more susceptible to degradation than chlorophyll *b*, the extract of cooked green pea distributed mostly pyropheophytin a (Fig. 5). The magnesium-free derivatives were eluted with retention values around that of β-carotene without overlapping. It should be mentioned that the chromatograms were monitored at 650 nm to exclude the carotenoid pigments while assisting in detecting the chlorophylls and their derivatives.

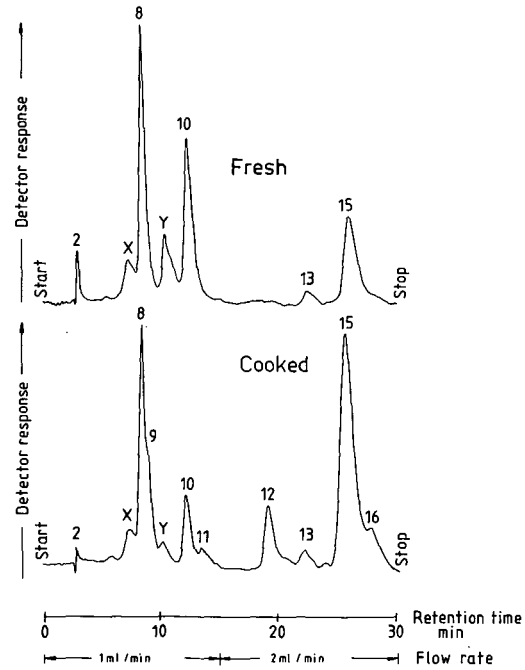

Fig. 5. Separation of chlorphyll-type pigments of fresh and cooked green pea on a Chromsil C_{18} column eluted with acetonitrile–methanol–ethyl acetate (53:40:7). Detection at 650 nm.

TABLE II

β-CAROTENE CONTENTS (mg PER 100 g OF EDIBLE FOOD) OF VEGETABLES DETERMINED BY HPLC

Vegetable	*No. of determinations*	*β-carotene (mean ± S.D.)*	*C.V. (%)*
Green pea (Jof)	4	0.22 ± 0.01	5.0
Green pepper:			
Bell-boy	5	0.88 ± 0.03	3.4
Szegedi-20	5	1.44 ± 0.04	2.8
Angeli emlike	5	1.56 ± 0.07	4.4
Cucumber (Aminex F-1)	4	1.15 ± 0.03	2.6
Lettuce (local)	6	3.12 ± 0.16	5.1
Spinach (local)	6	6.11 ± 0.20	3.3
Celery (local)	6	15.45 ± 0.62	4

Table II shows the determination of β-carotene in vegetables. The results indicate that the β-carotene content of different vegetables varies over a fairly wide range, depending on variations attributable to cultivars and growing location. It should be mentioned that the relatively high content of β-carotene in celery leaves indicates the high nutritive value of this product. The quantitative distribution of xanthophylls, chlorophylls and carotenes in several raw and processed vegetables will be described in another paper.

CONCLUSION

The simultaneous separation and detection of the major carotenoids, chlorophylls, and magnesium- and phytol-free derivatives of chlorophylls was achieved under isocratic conditions with an elution time of less than 35 min by using a Chromsil C_{18} column with acetonitrile–methanol–ethyl acetate (53:40:7) as the eluent with a stepwise increase in flow-rate. This method is simple and applicable to the determination of a wide variety of photosynthetic pigments.

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Note

Isotachophoretic separation of cyano complexes of noble metals using ion-pairing equilibrium in water–acetonitrile solvent

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Noble metals in the form of cyano complexes are widely used for metal plating in industry and for ornaments and many methods for their determination have been reported, *e.g.*, spectrophotometry[1], chromatography[2–4], atomic absorption spectrometry[5] and inductively coupled plasma atomic emission spectrometry[6].

Recently, we reported an isotachophoretic method for the determination of heavy metal ions as cyano complexes[7]. In this method, the metal cations injected were converted into cyano complexes by reaction with cyanide in the terminating electrolyte in the migration column and migrated as negatively charged ions. The effective mobility of each cyano complex was controlled by complex-forming equilibria between the metal ions and the cyanide in the terminating electrolyte and by the pH of the leading electrolyte. However, application of this migration system to the separation of noble metal ions was difficult because most noble metal ions form very stable or inert complexes with cyanide, which makes it impossible to control the mobilities of such complexes only by complex-forming equilibria with the terminating ion. It is known to be effective to use the interaction of a counter ion or additive in the leading electrolyte in order to control the mobility[8,9]. Therefore, in this work, we attempted to use ion-pairing equilibria to separate the cyano complexes of noble metal ions, such as palladium, platinum, gold, silver and nickel. It was established that using ion-pairing equilibria between these complex anions and a bivalent complex counter cation in water–acetonitrile is effective in achieving the required separation.

EXPERIMENTAL

Apparatus

A Model IP-1B capillary tube isotachophoretic analyser (Shimadzu, Kyoto, Japan), equipped with a potential gradient detector and a column system consisting of a PTFE preseparation capillary (50 × 1.0 mm I.D.) and a PTFE analytical capillary column (150 × 0.5 mm I.D.), was used. The current was stabilized at 50 μA after migration at 100 μA for 8–10 min. The capillary tube was filled with the leading and the terminating electrolytes by pressure of nitrogen gas.

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Reagents

Hydrochloric acid, tris(hydroxymethyl)aminomethane (Tris), tetradecyldimethylbenzylammonium chloride (Zephiramine) and poly(vinyl alcohol) were of analytical-reagent grade from Wako (Osaka, Japan) and were used without further purification.

Stock solutions of cyano complexes of nickel(II), platinum(II), silver(I) and gold(I) were prepared by dissolving potassium tetracyanoniccolate(II), barium tetracyanoplatinate(II), potassium dicyanoargenate(I) and potassium dicyanoaurate(I) (Wako) in water. Potassium tetracyanopalladate(II) and tris(1,10-phenanthroline)-iron(II) chloride were synthesized as described below[10].

Synthesis of potassium tetracyanopalladate(II). Palladium chloride (1 g) was dissolved in 70 ml of 0.01 *M* hydrochloric acid and 1 *M* potassium cyanide solution was added to it until palladium cyanide precipitated. The precipitate was filtered off and dissolved in potassium cyanide solution. After evaporation, the residual solid was recrystallized from water.

Synthesis of tris(1,10-phenanthroline)iron(II) chloride. Iron(II) chloride and 1,10-phenanthroline in a molar ratio of 1:3 were dissolved in methanol and the solution was filtered. Acetone was added and the required compound precipitated. The precipitate was recrystallized from water.

Electrolytes

The operational electrolyte system is shown in Table I. Tris–HCl buffer, the molar ratio of which was constant at 2.5:1, was used as the leading electrolyte at a pH of *ca.* 8.4. The concentration of the leading ion was constant at 6 m*M*. A mixture of acetonitrile and water was used as the solvent to prevent the precipitation of ion pairs and to adjust the ion-pairing equilibria.

RESULTS AND DISCUSSION

In a previous paper[7], we reported that six heavy metal ions could be separated by isotachophoresis by using complex-forming equilibria with cyanide as the terminating ion. However, cyano complexes of noble metal ions which have high stability constants could not be detected because of their mixing with the leading zone owing to their high mobilities. These complexes would be detectable by adding an ion-pairing reagent to leading electrolyte. When tris(1,10-phenanthroline)iron(II) or Ze-

TABLE I

OPERATING SYSTEM

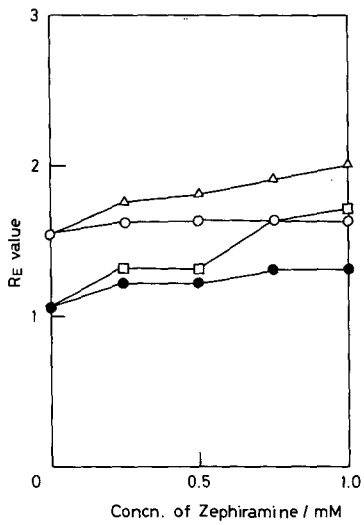
	Leading electrolyte	*Terminating electrolyte*
Anion	6 m*M* Cl^-	10 m*M* CN^-
Counter ion	$TrisH^+$[a]	K^+
Co-counter ion	Zephiramine, $Fe(phen)_3^{2+}$	
Additive	0.01% poly(vinyl alcohol)	50 m*M* $Ba(OH)_2$
Solvent	Water–acetonitrile	Water

[a] Tris:HCl = 2.5:1.

phiramine was used as the ion-pairing reagent, the effective mobilities of these cyano complexes decreased owing to the ion-pairing equilibria and the zone could be detected. Then the use of a mixed solvent (water–acetonitrile) was necessary to prevent precipitation of the ion-pair.

The influence of the concentration of the ion-pairing reagents on the R_E values (ratio of the potential gradient of the sample zone to that of the leading zone) of the cyano complexes is shown in Figs. 1 and 2. The effective mobilities of the cyano complexes decreased as the concentration of Zephiramine and tris(1,10-phenanthroline)iron(II) increased and the differences in the mobilities of the cyano complexes increased. With 1 m*M* tris(1,10-phenanthroline)iron(II) the differences in the mobilities of the complexes were greater than those using zephiramine.

The presence of acetonitrile influences the effective mobilities of the cyano complexes. The effect of acetonitrile on the effective mobilities with 1 m*M* tris(1,10-phenanthroline)iron(II) is shown in Fig. 3. At 0–5% (v/v) of acetonitrile, nickel and platinum or silver and gold could not be separated. When the concentration of acetonitrile was greater than 10%, the difference in mobilities increased and separation could be achieved. This effect of acetonitrile is related to the formation of ion pairs. The interaction of an ion pair is generally stronger in aqueous than in organic solutions. The interaction of the cyano complexes and the ion-pair reagent in aqueous solution is too strong to cause sufficient differences in the mobilities of the cyano complexes. However, by adding acetonitrile, this interaction becomes weaker so that each cyano complex can be separated. This was confirmed by the fact that the conductivity of the ion pair was greater in 20% acetonitrile solution than in aqueous solution using the ion pair of tris(1,10-phenanthroline)iron(II) and dicyanoargentate.



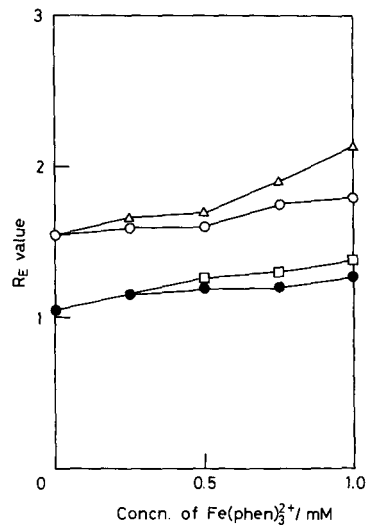


Fig. 1. Effect of concentration of zephiramine in leading electrolyte on R_E values of cyano complexes. Concentration of acetonitrile, 20% (v/v). ● = Nickel; □ = platinum; ○ = silver; △ = gold.

Fig. 2. Effect of concentration of tris(1,10-phenanthroline)iron(II) in leading electrolyte on R_E values of cyano complexes. Concentration of acetonitrile, 20% (v/v). Symbols as in Fig. 1. phen = 1,10-Phenanthroline.

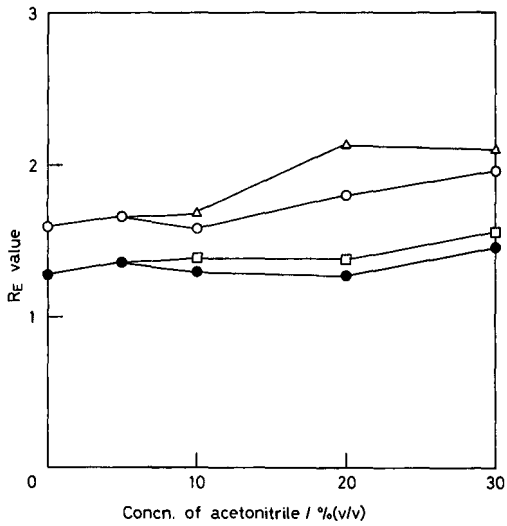


Fig. 3. Effect of concentration of acetonitrile in leading electrolyte on R_E values of cyano complexes. Concentration of tris(1,10-phenanthroline)iron(II), constant at 1 m*M*. Symbols as in Fig. 1.

An isotachopherogram of four cyano complexes with 20% acetonitrile solution containing 1 m*M* tris(1,10-phenanthroline)iron(II) is shown in Fig. 4. The order of their mobilities was nickel > platinum = palladium > silver > gold. Although nickel and platinum, or silver and gold, had very close mobilities in a migration system without an ion-pair reagent, the difference between the mobilities of the complexes increased as a result of ion-pairing equilibria and they could be separated. However, the cyano complexes of palladium and platinum showed the same isotachophoretic behaviour and they could not be separated even utilizing ion-pairing equilibria.

Under these conditions the calibration graphs of each metal ion were linear in the range 1–5 nmol. The reproducibilities of the zone length at 1 and 5 nmol are given

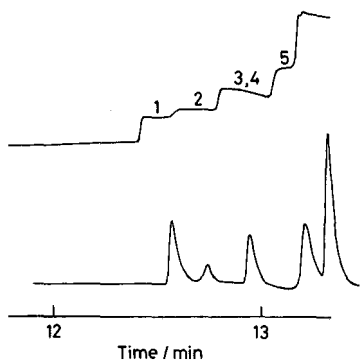


Fig. 4. Isotachopherogram of cyano complexes. Leading electrolyte, 20% (v/v) acetonitrile solution containing 1 m*M* tris(1,10-phenanthroline)iron(II). 1 = Nickel; 2 = platinum; 3 = hydrogencarbonate; 4 = silver; 5 = gold.

TABLE II

PRECISION OF ZONE LENGTH

Metal ion	*Amount (nmol)*	*Zone length (s)*	*R.S.D.*[a] *(%)*	*Number of determinations*
Nickel	1	5.2	5.9	4
	5	24.0	2.6	5
Platinum	1	5.1	1.1	4
	5	21.6	1.5	5
Silver	1	13.3	0.4	4
	5	26.1	0.6	5
Gold	1	3.6	4.2	4
	5	17.1	2.1	5

[a] Relative standard deviation.

in Table II. Except for 1 nmol of nickel, the reproducibilities were below 5%. The zone of silver became longer because of the formation of a mixed zone with hydrogencarbonate. The zone of hydrogencarbonate did not disappear completely on addition of 10 m*M* barium chloride to the sample solutions. A migration system of low pH is necessary to separate the silver zone from the hydrogencarbonate zone. However, when 10 m*M* barium chloride was added to all samples, the calibration graph of silver was linear and the slope was the same as that of monovalent dicyanoaurate. Therefore, from the linearity of the calibration graph, the zone length of hydrogencarbonate could be calculated.

This migration system could be applied not only to cyano complexes but also to metal chloride solutions as sample. Noble metal ions injected were converted into cyano complexes by reaction with cyanide as the terminating ion and could migrate as the anion. With the present method, it was found that the use of ion-pairing equilibria was effective for the separation of cyano complexes of noble metal ions.

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CHROM. 21 421

Note

Use of cyclodextrins in isotachophoresis

VIII. Two-dimensional chiral separation in isotachophoresis

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Many factors influence the separation efficiency of isotachophoresis (ITP)[1]. Modification of the leading electrolyte (LE) by non-polar complex-forming agents, cyclodextrins (CDs) proved to be an important method for the separation of different types of structurally related and/or isomeric compounds[2–9]. In previous contributions[10–12] we dealt with the use of CDs as enantioselective selectors added to the LE for the chiral separation of optical isomers in a device equipped with a one-column separation compartment. A device with coupled columns offers the possibility of performing one-run, two-step separations in two columns filled with LE of different composition[13].Mixtures of chiral substances of different structure usually cannot be resolved using one type of enantioselector only. In some instances it is possible to use mixtures of two or more selectors in the LE.

In this study we developed a two-dimensional chiral separation method that can be used with commercially available column-coupling equipment, both capillaries of which are filled separately with LE containing CDs as chiral selectors.

EXPERIMENTAL

Chemicals

Distilled water deionized with a Laboratory Water System XLDRO 1002 apparatus (Liquipure Europe, U.K.) was used for the preparation of the electrolyte solution and of the solutions of model compounds. All chemicals were of the highest quality commercially avialable: acetic acid, sodium acetate and oxalic acid were from Merck (Darmstadt, F.R.G.), β-alanine (β-Ala) from Serva (Heidelberg, F.R.G.); Natrosol 250 HR (hydroxyethylcellulose, HEC) from Hercules (Wilmington, DE, U.S.A.), Zerolit DM-F indicator from BDH (Poole, U.K.), γ-cyclodextrin (γ-CD) from Astec (Whippany, U.S.A.) and heptakis (2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) from Chinoin (Budapest, Hungary).

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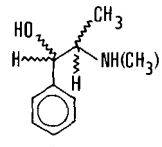
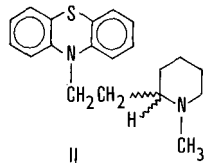
TABLE I

STRUCTURAL FORMULAE OF THE MODEL COMPOUNDS



I



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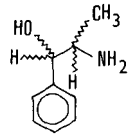
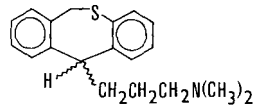


III



IV

Compound	*Name*
I, (+)- or (−)-	(+)- or (−)-pseudoephedrine; (1*S*,2*S*)- or (1*R*,2*R*)-2-methylamino-1-phenylpropanol
II, (+)- or (−)-	(+)- or (−)-thioridazine; (*R*,*S*)-10-[2-(1-methyl-2-piperidyl)ethyl]-2-methylthiophenothiazine
III, (+)- or (−)-	(+)- or (−)-norpseudoephedrine; (1*S*,2*S*)- or (1*R*,2*R*)-2-amino-1-phenylpropanol
IV, (+)- or (−)-	(+)- or (−)-hydrothiadene = (*R*,*S*)-11-(3-dimethylaminopropyl)-6,11-dihydrodibenzo(*b*,*e*)thiepin

Cyclodextrins and Natrosol 250 HR solutions were purified using Zerolit DM-F mixed bed ion-exchange resin. The solutes investigated were obtained from Research Institute for Pharmacy and Biochemistry (Prague, Czechoslovakia). Their formulae and numbering are given in Table I. Stock sample solutions were prepared by dissolving substances I and III in water (1 mg ml^{-1}) and substances II and IV in 5 m*M* oxalic acid (1 mg ml^{-1}) and were stored in dark bottles in a refrigerator.

Methods

Isotachophoretic experiments were performed using a ZKI-001 column-coupling isotachophoretic analyser (Institute of Radioecology and Applied Nuclear

TABLE II

ELECTROLYTE SYSTEMS AND CONDITIONS FOR ITP

Parameter	*Conditions*
Leading electrolyte	10 m*M* sodium acetate containing 0.08% HEC with acetic acid to pH 5.47
Terminating electrolyte	10 m*M* β-Ala
Capillary	1st column: 170 mm × 0.8 mm I.D. 2nd column: 170 mm × 0.3 mm I.D.
Current	In 1st column, 200 μA (800 s); for detection 100 μA In 2nd column, 50μA

Techniques, Plant for Development and Production of Nuclear Instruments, Spišská Nová Ves, Czechoslovakia) equipped with two polytetrafluoroethylene capillary columns with independent conductivity detectors and sample valve (volume 30 μl). The operating conditions are given in Table II.

The optical rotation of optically enriched mixtures of enantiomers were measured with a model 241 polarimeter (Perkin-Elmer, Norwalk, CT, U.S.A.). The ITP separation pattern of enantiomers of compounds II and IV was determined indirectly by comparison of ITP and optical rotation measurements for optically enriched samples obtained by selective precipitation of racemates with γ-CD on the micropreparative scale.

RESULTS AND DISCUSSION

The compounds in Table I were used for the preparation of four-component model mixtures (two pairs of appropriate enantiomers). Preliminary experiments indicated that I and III could be enantioselectively separated with DM-β-CD-modified LE and II and IV could be enantioselectively separated in γ-CD-containing LE.

The requirements for the chiral separation of solute mixtures of I + II and/or III + IV racemates are much more demanding on the experimental technique. It

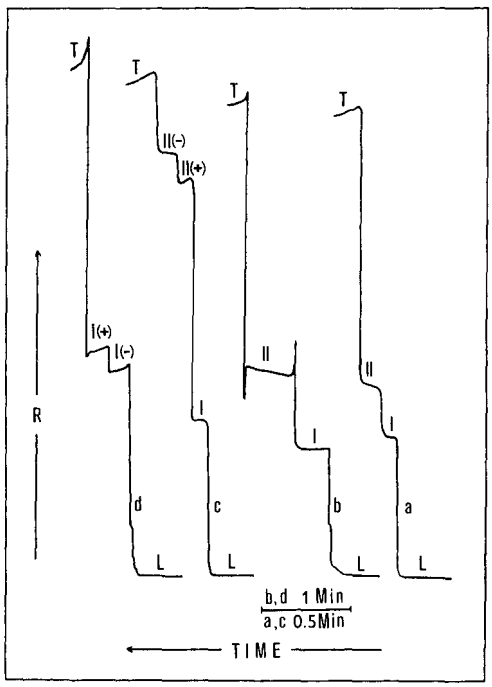


Fig. 1. ITP separation of the optical isomers of compounds I and II (a and b) without CD, (c) with 5 mM γ-CD and (d) with 10 mM DM-β-CD in the leading electrolyte with the two-column system (a and c) in the first capillary and (b and d) in the second capillary. R = response of detector; L and T = leading and terminating zones, respectively. Amount of sample introduced: (I) 1.07 μg and (II) 3.21 μg, in all measurements.

cannot be achieved in one column filled with a mixture of the two CDs at concentrations found to be most effective for the resolution of single racemates. The main problem results from the strong non-effective complexation[14] of II and IV, the DM-β-CD complexes of which are lost in the terminating electrolyte zone.

The most effective solution to this problem is to apply a two-column system in which each separation compartment is filled with one type of CD-modified LE. Practical examples of the two-dimensional chiral separation of mixtures of I and II and of III and IV racemates are illustrated in Figs. 1 and 2, respectively.

The non-chiral separation of the solutes in the two separation compartments filled with CD-free LE is shown in traces (a) and (b). The addition of γ-CD to the first and DM-β-CD to the second compartment, at the optimum concentrations given in the legends, leads to complete enantioselection in one experimental run. The complete chiral separation of solutes II and IV by means of γ-CD is shown in traces (c). Compounds I and III became almost uncomplexed and were completely unresolved. Different separation patterns were observed in the second compartment filled with DM-β-CD. All the compounds investigated were substantially complexed. The strong retardation of II and IV led to their disappearance in the zone of the terminat-

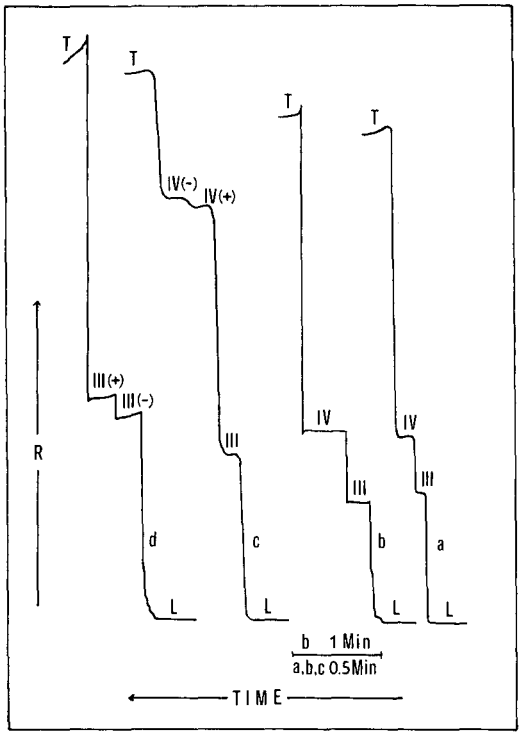


Fig. 2. ITP separation of the optical isomers of compounds III and IV (a and b) without CD, (c) with 15 mM γ-CD and (d) with 10 mM DM-β-CD in the leading electrolyte with the two-column system. Amount of sample introduced: (III) 0.86 μg in (a) and (b) and 0.66 μg in (c) and (d); (IV) 3.34 μg in (a) and (b) and 2.63 μg in (c) and (d). Other details as in Fig. 1.

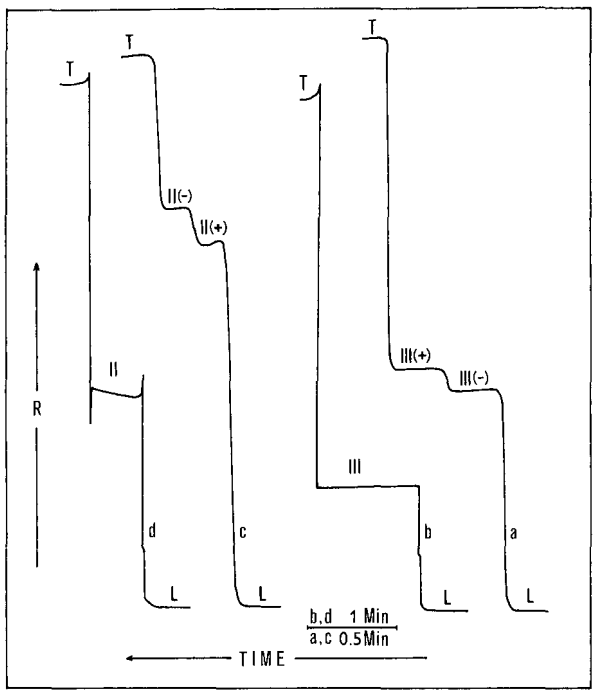


Fig. 3. Demonstration of instability of CD–enantiomer complexes of compounds II and III. The first capillary was filled with (c) 5 m*M* γ-CD and (a) 10 m*M* DM-β-CD modified LE, respectively. Amount of sample introduced: (II) 3.33 μg and (III) 3.00 μg. Other details as in Fig. 1.

ing electrolyte. In contrast, the interaction of solutes I and III with the CD led to a significant resolution of their optical isomers.

In order to examine the stability of the CD complexes formed, experiments were also carried out with the two-column system in which the first capillary was filled with CD-modified LE and the second did not contain CD in the LE. As illustrated in Fig. 3 and Table III, the CD complexes generally decompose during the migration

TABLE III

$(h_i)_{rel}$ VALUES OF COMPOUNDS I AND IV

$(h_i)_{rel} = (h_i - h_l) / (h_t - h_l)$, where h_i, h_l and h_t = step height of sample, leading electrolyte and terminating electrolyte, respectively.

Compound	*1st column (with CD)*		*2nd column (without CD)*
	(+)-Enantiomer	*(−)-Enantiomer*	*racemate*
I[a]	0.487	0.448	0.270
IV[b]	0.737	0.764	0.382

[a] LE with 10 m*M* DM-β-CD.
[b] LE with 15 m*M* γ-CD.

through the second CD-free capillary. This results in a lowering of the $(h_i)_{rel}$ values and combination of zones of the enantiomers resolved in the first column. It should be noted, however, that the fixed distance between the two detectors does not enable the half-life of decomposition of the CD complex to be determined accurately.

CONCLUSIONS

The two-dimensional ITP system described, using different types of CDs for modification of the LE, proved useful for the investigation of complicated mixtures of racemates. The main advantages are a shorter separation time comparing with two runs with a one-column system filled gradually with an enantioselector, lower consumption of the samples and chiral selectors and the possibility of using two interfering selectors in one two-step separation.

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Note

Some data on the thin-layer and high-performance liquid chromatographic separation of some dithia[3.3]phanes

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Dithia[3.3]phanes are important intermediates in the synthesis of the strained [2.2]phanes. Phanes have been characterized by chromatographic techniques in only a limited number of cases; high-performance liquid chromatographic (HPLC) and gas chromatographic investigations have been carried out on [2.2]naphthalenophane derivatives and [3.3]phanes[1]. Some additional HPLC separations on a chiral stationary phase have been performed on several [2.2]phanes with heteroatoms (S, O, N) in the bridge[2].

In this work we established conditions for the thin-layer chromatographic (TLC) and HPLC separation of fifteen dithia[3.3]phanes. Earlier analogous conditions had been elaborated for some of these compounds on silica and alumina[3,4]; here the stationary phase considered was silica with a modified surface (RP_{18}) in both techniques. The influence of Ag^+ ions in the mobile phase on the separation of the selected dithia[3.3]phanes was studied. We are aware that compounds **1–15** are never encountered as products in the same reaction, but the established analytical conditions can prove helpful when separating some stable isomers of other dithia[3.3]phanes, or when purifying this type of compound by preparative liquid chromatography.

EXPERIMENTAL

Compounds **1–15** were obtained from the respective di(bromomethyl)naphthalenes and di(mercaptomethyl)benzene derivatives (**1–4**[5], **5–12**[4] and **13–15**[6]) by means of the high-dilution technique, and they were further separated chromatographically.

TLC

The compounds were separated on ready-made chromatographic plates covered with modified-surface silica ($RP_{18}F_{254}S$; Art.No.15423, Merck, Darmstadt, F.R.G.). The mobile phases were methanol–water (98:2, 95:5 and 90:10, v/v) and acetonitrile–methanol (50:50, v/v). Solutes were spotted in aliquots of 5 μl as 1% benzene solutions, with UV detection at 254 nm.

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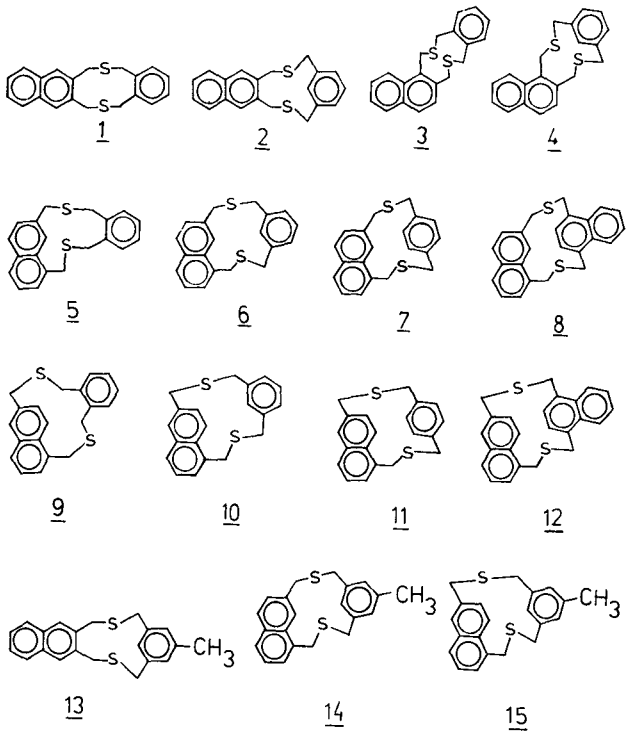

HPLC

The compounds were separated using a liquid chromatograph (Laboratorni pristroje, Prague, Czechoslovakia) composed of an HPP 5001 pump, an LCD 2563 UV–VIS detector equipped with a 254-nm filter, a TZ-4620 recorder and a CI-100

TABLE I

R_F VALUES OF COMPOUNDS **1–15**

Compound	*Methanol–water (v/v)*			*Acetonitrile–water (50:50, v/v)*
	98:2	*95:5*	*90:10*	
1	0.39	0.31	0.18	0.55
2	0.35	0.26	0.17	0.53
3	0.38	0.28	0.20	0.58
4	0.34	0.25	0.17	0.54
5	0.35	0.24	0.18	0.54
6	0.39	0.29	0.23	0.59
7	0.45	0.34	0.24	0.63
8	0.40	0.30	0.19	0.64
9	0.38	0.29	0.18	0.56
10	0.38	0.29	0.18	0.58
11	0.39	0.30	0.18	0.58
12	0.36	0.27	0.16	0.58
13	0.32	0.24	0.13	0.51
14	0.38	0.30	0.17	0.56
15	0.36	0.27	0.14	0.55

TABLE II

HPLC RETENTION TIMES (min) OF COMPOUNDS **1–15**

Compound	*Methanol–water (v/v)*				
	100:0	*90:10*	*85:15*	*80:20*	*75:25*
1	2.07	5.89	10.57	20.71	33.63
2	2.21	6.28	11.18	20.80	36.54
3	2.03	5.39	9.67	17.28	29.30
4	2.20	6.31	11.27	20.20	36.03
5	2.15	5.96	10.76	18.80	31.32
6	1.99	4.73	7.45	14.02	22.59
7	1.83	3.94	5.74	10.45	18.89
8	1.96	4.63	7.12	14.26	25.70
9	2.08	5.00	7.87	15.51	27.00
10	2.08	4.93	7.56	14.54	24.54
11	2.10	4.98	7.52	14.67	23.82
12	2.12	5.51	9.83	18.83	31.61
13	2.42	7.80	15.35	29.12	52.91
14	2.15	5.60	9.46	20.2	38.00
15	2.24	5.80	9.49	20.0	38.0

integrator. Separations were carried out on a glass column (150 mm × 3.6 mm I.D.) filled with Separon SGX-C18 (octadecylsilica) of particle diameter 5 μm (Laboratorni pristroje) with a mobile phase flow-rate of 0.8 cm^3/min. Solutes were applied as 0.1% solutions in tetrahydrofuran. The mobile phases were methanol–water (100:0, 90:10, 85:15, 80:20 and 75:25, v/v); compounds **5–12** were also separated using methanol–water (80:20, v/v) with $2 \cdot 10^{-2}$ *M* Ag^+ ions added.

RESULTS AND DISCUSSION

The R_F values obtained by TLC are given in Table I and the HPLC retention times in Table II. Table III gives the results obtained for compounds **5–12** by HPLC with Ag^+ ions in the mobile phase.

TABLE III

EFFECT OF SILVER IONS ON THE HPLC RETENTION TIMES (min) OF COMPOUNDS **5–12**

Compound	*Methanol–water (80:20, v/v)*	*Methanol–water (80:20, v/v) + $2 \cdot 10^{-2}$ M Ag^+*
5	18.80	3.92
6	14.02	5.13
7	10.45	10.97
8	14.26	8.42
9	15.51	8.55
10	14.54	16.33
11	14.67	7.46
12	18.83	8.94

In TLC the R_F values decrease and in HPLC the retention times increase with increasing concentration of water in the mobile phase. An increase in the proportion of water also results in an enhanced resolving power of the HPLC system.

The data in Table II show that replacement of the hydrogen atom by a methyl group in the smaller aromatic fragment of a given dithia[3.3]phane results in a longer retention. The ^{1}H NMR spectra established that this replacement induces no conformational changes in the molecules in solution, and for this reason the longer retention can simply be ascribed to an increased effective molecular volume, responsible for the relative retardation.

Complexation of the dithia[3.3]phane molecules with Ag^+ ions in most instances results in shorter retention times and in an enhanced resolution of the chromatographic system.

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CHROM. 21 404

Note

Some new by-products in primary structure analysis with dimethylaminoazobenzene isothiocyanate as an aid in the thin-layer chromatographic identification of residues

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4-N,N-Dimethylaminoazobenzene 4′-isothiocyanate (DABITC) is a sensitive chromophoric reagent for protein sequence analysis[1]. For use in N-terminal analysis it can be used as the only coupling reagent[2]. However, the low coupling yield with DABITC makes the use of a second coupling reagent, phenyl isothiocyanate (PITC), necessary in sequencing procedures[3]. Both high-performance liquid chromatography[4,5] and thin-layer chromatography (TLC)[3,6] have been employed to identify dimethylaminoazobenzene thiohydantoins (DABTH).

Manual sequencing with DABITC and PITC and identification of the residues with TLC is an inexpensive method, as it does not require much instrumentation. A serious drawback, however, is the difficulty in identifying some of the DABTH derivatives. The hydrophobic residues are clustered and leucine and isoleucine co-chromatograph on polyamide thin layers[3]. Leucine and isoleucine can be separated on silica gel, but this procedure reduces the sensitivity of the method[6]. In addressing the problem, Von Bahr-Lindström *et al.*[7] published some characteristic TLC by-product patterns that are very useful in identifying several residues, including isoleucine and leucine.

We have found some additional by-product spots on polyamide thin layers that make the identification of especially histidine and lysine much easier. In this paper we describe the chromatographic patterns that can be obtained for proline, alanine, lysine and histidine. A reason for the formation of the by-products is discussed.

EXPERIMENTAL

Chemicals

Heptane, ethyl acetate, trifluoroacetic acid (TFA) and PITC (Fluka, for sequential analysis) were used without further purification. Pyridine (Fluka, puriss. p.a.) was redistilled twice. *n*-Butyl acetate (Merck, analytical-reagent grade) was redistilled and DABITC (Fluka) was recrystallized from boiling acetone.

The peptides used were L-lysyl-L-serine and L-histidyl-L-aspartic acid (Sigma). Peptides were also obtained during primary structure determination of carbonic anhydrase from tiger shark.[8]

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Amino acid sequence analysis

The sequencing protocol described by Chang *et al.*[3] was used, except for the conversion reaction, where 50 μl of 50% (v/v) TFA was used. DABTH derivatives were separated on 3 × 3 cm polyamide sheets (F 1700, Schleicher & Schüll) in a two-solvent system. For the first dimension acetic acid–water (1:2, v/v) and for the second dimension toluene–*n*-hexane–acetic acid (2:1:1, v/v) were used as solvents as described by Chang *et al.*[3].

RESULTS AND DISCUSSION

In addition to the by-product patterns reported by Von Bahr-Lindström *et al.*[7], we have found several new by-products during TLC identification of the DABTH derivatives. The chromatographic patterns shown in Fig. 1 are characteristic of the respective amino acids.

For alanine we always obtain a red spot, which appears above and to the right of the red spot for DABTH-alanine. Proline gives a red spot to the right of the red DABTH-proline spot. When lysine is the N-terminal amino acid, there is a blue spot above and to the right of the point of sample application, whereas an N-terminal histidine gives a blue spot to the right of the sample application point.

The two latter spots have proved most useful for identification of the respective amino acids. Histidine residues may be difficult to identify for two reasons. First,

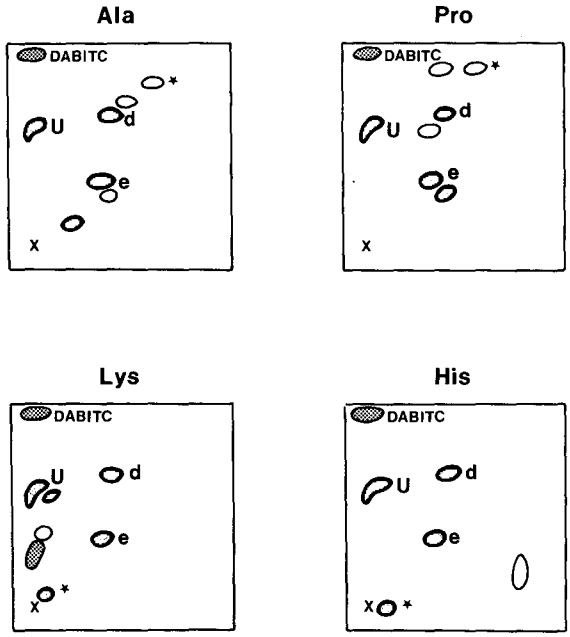

Fig. 1. Chromatographic patterns obtained in the DABITC degradation of alanine, proline, lysine and histidine. Red spots are marked as open, blue as filled and purple as hatched areas. The spots marked d and e are the markers DABTC-diethylamine and DABTC-ethanolamine, U is a thiourea product formed by the coupling of PITC with hydrolysed DABITC[3]. The new by-product spots are labelled with asterisks, and the other spots were described by Chang *et al.*[3] and Von Bahr-Lindström *et al.*[7].

DABTH-histidine is difficult to distinguish from DABTH-arginine and second there is often a reddish streak on the right edge of the TLC plate that might obscure or be mistaken for DABTH-histidine. The new by-product spot for histidine makes the identification of histidine residues much more reliable. With lysine the previously reported chromatographic pattern might be obscured by a bluish streak from the point of sample application. The newly discovered spot, which we always obtain in high yield, is easily detected.

If the dimetylaminoazobenzenethiazoline (DABTZ) derivatives are extracted with newly distilled *n*-butyl acetate, the by-product spots have a low intensity compared with the DABTH spots, but with time the by-products increase at the expense of the DABTH derivatives. If the *n*-butyl acetate is redistilled, the relative amount of by-products decreases. When the volume of *n*-butyl acetate used is decreased, the relative amount of by-products also decreases. The smaller the amount of peptide being sequenced, the higher is the ratio of by-products to DABTH derivatives. Apparently the formation of by-products depends on the purity and amount of the *n*-butyl acetate used. Taken together, this suggests that the by-products are formed in a constant amount by reaction of an impurity in the *n*-butyl acetate with the DABTZ derivative, the DABTH derivative or an intermediate.

Our conclusion is that in addition to the by-products reported by Von Bahr-Lindström *et al.*[7], the by-product spots of especially histidine and lysine residues are very useful in their identification on polyamide TLC plates.

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Note

Simultaneous determination of nitrofuran derivatives in various animal substrates by high-performance liquid chromatography

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Nitrofuran derivatives have been used for more than 30 years in veterinary medicine, alone or in combination with other drugs. Nitrofurans are metabolized extensively[1], but the metabolic pathways have still not been elucidated[2]. Since the demonstration that nitrofurans are mutagenic and (pro)carcinogenic, their use has been strictly regulated in many countries. Recently, the EEC has recommended a tolerance level of 1–2 ppb[a] for the parent nitrofurans[3,4] in edible tissues of slaughtered animals. Hitherto, drug instability and the lack of sensitive detection methods has made the detection of nitrofurans at the 1 ppb level complicated.

A previous study[1] revealed that degradation of furaltadone and nitrofurazone dissolved in calves' urine could be prevented by dilution of the urine with 1 *M* phosphate buffer (pH 5.0) containing 0.2% sodium azide (enzyme blocker) with immediate storage at −20°C and taking strict precautions to protect them from light.

This study describes an *in vitro* tested stabilization procedure that prevents degradation of nitrofurans in organic tissue and a rapid, sensitive and selective high-performance liquid chromatographic (HPLC) method for the simultaneous determination of four nitrofuran derivatives (nitrofurantoin, nitrofurazone, furazolidone and furaltadone) in plasma, urine and body tissues at the 1 ppb level.

The application of the procedure to the routine determination of nitrofuran derivatives is demonstrated.

EXPERIMENTAL

Apparatus and chromatographic conditions

The HPLC equipment consisted of an SF 400 pump (Kratos, Rotterdam, The Netherlands), a Rheodyne 7125 injector with a 100-μl sample loop, a Spectroflow 783 detector (Kratos) and a Shimadzu CR-3a integrator. Separations were carried out with a Zorbax CN (5 μm) column (250 × 4.6 mm I.D.) (DuPont, Wilmington, DE, U.S.A.). A guard column (50 × 2.1 mm I.D.) tap-packed with a pellicular reversed phase (Chrompack, Middelburg, The Netherlands) was fitted in front of the analytical column.

The eluent was prepared by mixing 600 ml of 0.01 *M* sodium acetate buffer (pH

[a] Throughout this paper, the American billion (10^9) is meant.

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5.0) with 400 ml of methanol. The eluent was degassed and filtered before use. The flow-rate was 1.50 ml/min, the analyses were performed at 20°C and the detection of the nitrofuran derivatives was performed at 365 nm with a detector setting of 0.001 a.u.f.s.

Chemicals and reagents

Furaltadone hydrochloride (Aesculaap, Boxtel, The Netherlands), nitrofurazone (Furazin; Stricker, Berne, Switzerland), furazolidone (Sigma, St. Louis, MO, U.S.A.) and nitrofurantoin (Sigma) were used. Their structures are shown in Fig. 1.

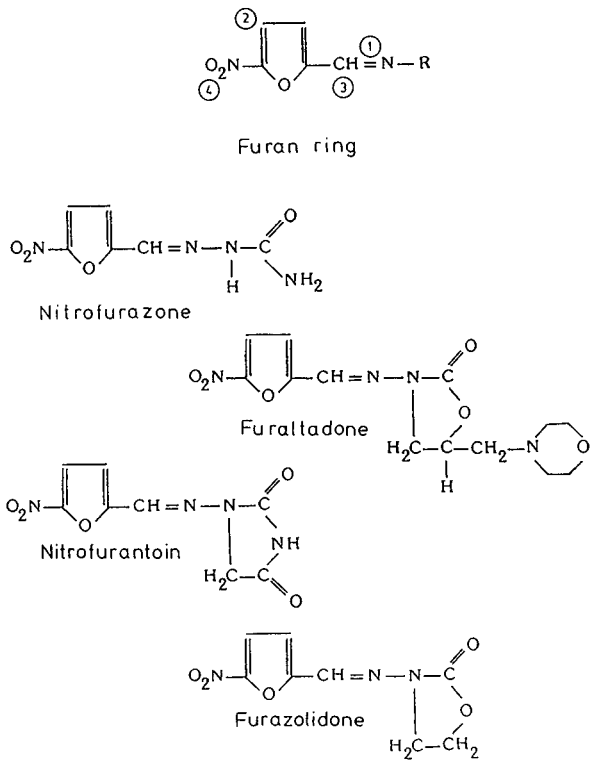

Fig. 1. Structure of nitrofurans and of furaltadone, furazolidone, nitrofurantin and nitrofurazone.

Ethyl acetate (Uvasol), acetonitrile (Uvasol), dichloromethane (analytical-reagent grade) and *n*-hexane (analytical-reagent grade) were obtained from Merck (Darmstadt, F.R.G.). Phosphate buffer (0.67 *M*, pH 5.0) was prepared by dissolving 22.69 g of KH_2PO_4 and 0.64 g of $Na_2HPO_4 \cdot 2H_2O$ in 250 ml of distilled water.

Stability studies

The *in vitro* stability of the nitrofuran derivatives was studied in meat and liver of cows, swine and poultry. These tissues were refrigerated at 4°C for 1 day. Meat and

liver were homogenized with (i) physiological saline, (ii) 1 *M* phosphate buffer (pH 5.0) and (iii) 1.5 *M* KH_2PO_4 solution (pH 4.5) containing 0.2% sodium azide. Pools were spiked to a concentration of 50 ppb, kept in complete darkness and analysed at different times.

Preliminary studies revealed that degradation of the nitrofurans could be prevented by the last procedure. Therefore, the following preparation procedures were performed for the validation of the method.

Preparation of the samples

For urine, the sample was diluted 1:1 (v/v) with 1.5 *M* KH_2PO_4 containing 0.2% sodium azide. For plasma, 5 ml of 1.5 *M* KH_2PO_4 with 0.2% of sodium azide were added to a 50-ml sample. For edible tissues (bovine muscle and liver), the samples were homogenized (Waring blender) with 1.5 *M* KH_2PO_4 containing 0.2% sodium azide (1:2, w/v).

All samples were wrapped in tin-foil and immediately stored at −20°C until analysis.

Extraction procedures

During the extraction procedures the samples were protected from light.

Plasma and urine. To 10 ml of buffered plasma or urine, 10 ml of dichloromethane–ethyl acetate (1:1, v/v) were added, mixed by rotation (Heidolph rotator) at 20 rpm for 20 min and centrifuged at 4500 *g* for 10 min. An 8-ml volume of the organic layer was removed and evaporated to dryness at 50°C under a gentle stream of nitrogen, the residue was dissolved in 500 μl of phosphate buffer (0.67 *M*, pH 5.0) and 1 ml of *n*-hexane was added. After mixing and centrifugation, 100 μl of the aqueous phase were injected into the HPLC system.

Organic tissues. To 30 g of the homogenate, 20 ml of acetonitrile were added, mixed by rotation for 15 min and then centrifuged at 4500 *g* for 10 min. A 35-ml volume of the supernatant was transferred into another 50-ml tube and 5 ml of dichloromethane–ethyl acetate (1:1, v/v) were added, mixed by rotation for 15 min and centrifuged at 4500 *g* for 10 min. A 12-ml volume of the upper organic layer was removed, evaporated to dryness at 50°C under a gentle stream of nitrogen and 2 ml of *n*-hexane and 500 μl of phosphate buffer (0.67 *M*, pH 5.0) were added to the residue. After mixing and centrifugation, 100 μl of the aqueous phase were subjected to HPLC.

Quantification and confirmation

Quantification was carried out using calibration graphs obtained from spiked samples in the concentration range 1–100 ppb. The concentration of the drug was determined by comparison of peak areas or peak heights.

Interferences

To study interference, 1000 ppb of the antimicrobial agents chloramphenicol, sulphadimidine, sulphamethoxazole, sulphanilamide, sulphatroxazole, sulphathiazole, dapsone, oxytetracycline and carbadox were injected into the HPLC system.

RESULTS AND DISCUSSION

Homogenization of meat and liver with physiological saline or 1 *M* phosphate buffer (pH 5.0) showed rapid degradation of the nitrofurans. The degradation half-lives of nitrofurantoin, furazolidone, furaltadone and nitrofurazone bovine liver homogenates prepared in physiological saline were approximately 12, 12, 31 and 44 h, respectively (see Fig. 2). Different degradation half-lives for nitrofurans in the organs from different species should be emphasized. For sample in fresh goat and cattle livers the degradation half-life was about 13 min[5]. Decreasing the pH to 4.5 using 1.5 *M* KH_2PO_4 containing 0.2% sodium azide instead of saline or pH 5.0 buffer revealed an *in vitro* tested stabilization of the various nitrofuran derivatives for 24 h in edible tissues of swine, cows and poultry.

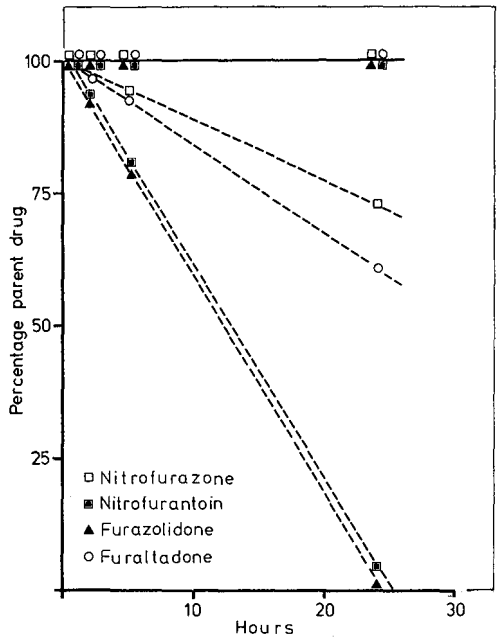

Fig. 2. Stabilization of nitrofurans in liver homogenates made with (broken lines) physiological saline and (solid line) 1.5 *M* KH_2PO_4 solution (pH 4.5) and 0.2% sodium azide.

Fig. 3 shows typical chromatograms of bovine plasma and meat spiked with nitrofurazone, nitrofurantoin, furazolidone and furaltadone. The four nitrofuran derivatives eluted as distinct peaks with retention times of 4.2, 4.6, 5.5 and 7.4 min, respectively. Calibration graphs for the four nitrofuran derivatives in plasma, urine, meat and liver (range 1–100 ppb) were linear ($r = 0.999$). The limit of detection was 1 ppb, yielding a detector response approximately equal to five times the detector noise.

Table I shows the recovery and reproducibility of the procedure for the nitrofuran derivatives in the various substrates. The method showed good precision

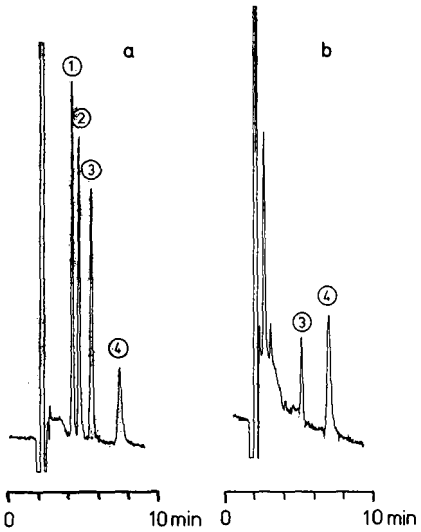

Fig. 3. Representative chromatograms (0.002 a.u.f.s.) of (a) plasma spiked with 10 ppb each of nitrofurantoin (1), nitrofurazone (2), furazolidone (3) and furaltadone (4) and (b) a meat sample with 1.13 ppb of furazolidone (3) and 4.78 ppb of furaltadone (4).

TABLE I

RECOVERY AND REPRODUCIBILITY OF THE PROCEDURES

Drug concentration range 1–100 ppb.

Sample	*Nitrofurazone*		*Nitrofurantoin*		*Furazolidone*		*Furaltadone*	
	Av.[a] *(%)*	*C.V.*[b] *(%)*	*Av.*[a] *(%)*	*C.V.*[b] *(%)*	*Av.*[a] *(%)*	*C.V.*[b] *(%)*	*Av.*[a] *(%)*	*C.V.*[b] *(%)*
Water								
Mean	91.5	2.6	90.2	3.0	97.7	2.6	87.2	3.4
S.D.	1.5	0.8	1.8	1.5	0.4	1.2	1.0	0.7
Linearity	0.9999		0.9999		0.9999		0.9999	
Bovine plasma								
Mean	69.7	3.0	72.8	3.9	89.7	2.7	88.1	4.0
S.D.	1.6	1.9	1.1	1.0	1.6	1.5	1.0	1.5
Linearity	0.9999		0.9999		0.9998		0.9998	
Bovine meat								
Mean	60.7	2.2	52.5	1.1	100.3	2.8	99.1	2.3
S.D.	0.4	1.5	0.5	0.4	2.2	2.2	1.9	1.5
Linearity	0.9998		0.9999		0.9998		0.9999	
Bovine liver								
Mean	60.7	2.0	52.3	1.8	99.9	1.9	99.1	1.5
S.D.	0.3	1.0	0.4	0.4	1.3	1.7	1.3	1.2
Linearity	0.9998		0.9999		0.9998		0.9998	

[a] Average of 40 determinations.

[b] Coefficient of variation.

and accuracy (coefficient of variation 1.1–4.0%). The combination of the low pH and sodium azide as enzyme blocker gave good stabilization and prevented *in vitro* degradation.

No interference in the chromatograms of nitrofurans was observed from chloramphenicol, sulphadimidine, sulphametoxazole, sulphanilamide, sulphatroxazole, sulphathiazole, dapsone and oxytetracycline; only carbadox eluted just before nitrofurazone. A diode-array detector can give good results for the identification of these two components.

Recent monitoring of 250 slaughtered diseased animals for nitrofuran residues with the method described revealed that the method was easy to perform and reliable for the simultaneous determination of nitrofuran derivatives in both body fluids and tissues.

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Note

Determination of chlorophenols in water by direct acetylation and solid-phase extraction

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(Received January 20th, 1989)

Phenols can be converted into their acetates directly in a dilute aqueous solution by a simple reaction with acetic anhydride[1–3]. The method used for the isolation and concentration of phenol acetates from water is usually repeated liquid–liquid extraction with subsequent concentration of the derivatives by solvent evaporation. Another enrichment method uses solid-phase extraction of phenols from acidified water samples, elution with an organic solvent, derivatization of phenols in the eluate and gas chromatographic (GC) analysis[4,5]. Retention data and mass spectra of chlorophenol acetates have been published[6].

The method described in this paper combines both of the above procedures. It consists of direct acetylation of chlorophenols in water, solid-phase extraction of chlorophenol acetates and GC analysis of the eluate. The advantages of this method are as follows: (1) non-ionogenic acetates may be isolated from water more easily than highly polar free chlorophenols; (2) derivatization in the aqueous solution is very fast and results in the formation of products suitable for direct GC analysis; and (3) in comparison with repeated liquid–liquid extraction, manipulations with large volumes of solvents are avoided.

EXPERIMENTAL

For solid-phase extraction, polypropylene cartridges packed with C_{18} reversed phase were used (Tessek) and/or Sep-Pak (Waters Assoc.). The volume of packing material in both cartridges used was about 0.5 ml.

A Varian 3700 gas chromatograph was used with flame ionization (FID) and/or electron-capture detection (ECD). Data were processed on an HP 3388 integrator (Hewlett-Packard). A wide-bore fused-silica capillary column (30 m × 0.5 mm I.D.) coated with RSL-150 (Alltech) was used for the separation of the chlorophenol acetates. No inlet splitter was used.

The GC parameters were as follows: carrier gas flow-rate [helium for FID and argon–methane (95:5) for ECD], 6 ml/min; temperature of injection block, 200°C; FID

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temperature, 260°C; ECD temperature, 315°C. The flow-rate of the make-up gas for ECD was 15 ml/min; no make-up gas was used for FID as the capillary column was led directly into the jet of the detector. The oven temperature was programmed from 140 to 225°C at 8°C/min for GC with ECD and from 60 to 220°C at 8°C/min for GC with FID.

Two different schemes were used for recovery measurements, the first being a simulated water sample and the other a 100% recovery standard:

Simulated water sample:

acetylation of chlorophenols in water
↓
solid-phase extraction
↓
removal of residual water from cartridge
↓
elution with organic solvent
↓
drying of eluate
↓
addition of internal standard
↓
gas chromatography

100% recovery standard:

acetylation of chlorophenols in organic solvent
↓
separation of organic layer
↓
drying of organic solvent
↓
addition of I.S.
↓
gas chromatography

Procedure

Tap water was spiked with different chlorophenols. Acetylation of chlorophenols was performed using a procedure similar to that described by Coutts *et al.*[1]. Sodium hydrogencarbonate (10 g) and acetic anhydride (1 ml) were added to the water sample (250 ml) and the mixture was shaken until evolution of carbon dioxide ceased. An aliquot of 100 ml of acetylated water sample was then pushed through the cartridge by means of a syringe. This enrichment step took 2 min, which corresponds to a flow-rate of 50 ml/min. Volumes of water up to 250 ml were tested and no breakthrough of the chlorophenol acetates tested was observed. The cartridge was then connected to a water evacuator in order to remove residual water, which took 2 min. Chlorophenol acetates trapped in the cartridge were desorbed with 1 ml of benzene. Desorption was performed stepwise three times using about 0.3 ml of solvent each time, so each portion corresponds to the cartridge dead volume. Each portion of desorption solvent was allowed to equilibrate in the cartridge for about 1 min. Benzene

was pushed through the cartridge by means of an all-glass syringe. The combined eluates were dried with a small amount of anhydrous sodium sulphate and, after addition of internal standard (*p*-bromophenol acetate), GC analysis was performed.

Solutions representing 100% recovery of chlorophenols from water were prepared by a method described by Renberg and Lindström[4]. An amount of chlorophenols corresponding to 100 ml of water sample was added in a methanolic solution to a reaction–extraction mixture containing 3 ml of 0.1 mol/l potassium carbonate, 1 ml of benzene and 50 μl of acetic anhydride. A test-tube containing the reaction mixture was shaken for 5 min. The aqeous phase was drained and the organic layer was dried with anhydrous sodium sulphate. The same amount of internal standard as was added to the cartridge eluate of water samples was also added to the organic phase of the reaction mixture. Then the solution was analysed by GC.

The recovery of the procedure was calculated from the ratio of the peak areas of chlorophenol acetates obtained from spiked water samples and from 100% recovery standards after correcting for the peak areas of the internal standard (I.S.) in the different GC runs.

RESULTS AND DISCUSSION

Recoveries were measured for various concentrations of chlorophenols in water and the results are summarized in Table I for FID and in Table II for ECD measurements. The recoveries were always higher than 90%.

Examples of chromatograms are shown in Figs. 1 and 2. Fig. 1 refers to the determination of chlorophenols in a spiked water sample for the concentrations given in the first column of Table I. Fig. 2 applies to a reference mixture corresponding to the concentrations given in the second column of Table II.

TABLE I

RECOVERY OF CHLOROPHENOLS FROM WATER BY GC WITH FID

Concentrations are given in parentheses.

Compound	*Recovery (%) (concentration in mg/l)*		
o-Chlorophenol	103.0 (5.51),	105.1 (0.330),	96.3 (0.165)
p-Chlorophenol	100.0 (2.72),	100.7 (0.163),	95.6 (0.082)
3,4-Dichlorophenol	98.2 (2.57),	99.1 (0.154),	93.9 (0.077)
2,4,5-Trichlorophenol	96.5 (2.44),	102.1 (0.146),	97.8 (0.073)
Pentachlorophenol	97.1 (4.80),	95.1 (0.288),	95.6 (0.144)

TABLE II

RECOVERY OF CHLOROPHENOLS FROM WATER BY GC WITH ECD

Concentrations are given in parentheses.

Compound	*Recovery (%) (concentration in μg/l)*	
o-Chlorophenol	92.0 (218),	96.2 (21.8)
3,4-Dichlorophenol	94.1 (218),	98.3 (21.8)
2,4,5-Trichlorophenol	101.2 (28.4),	105.2 (4.36)
Pentachlorophenol	93.0 (2.9),	92.1 (0.72)

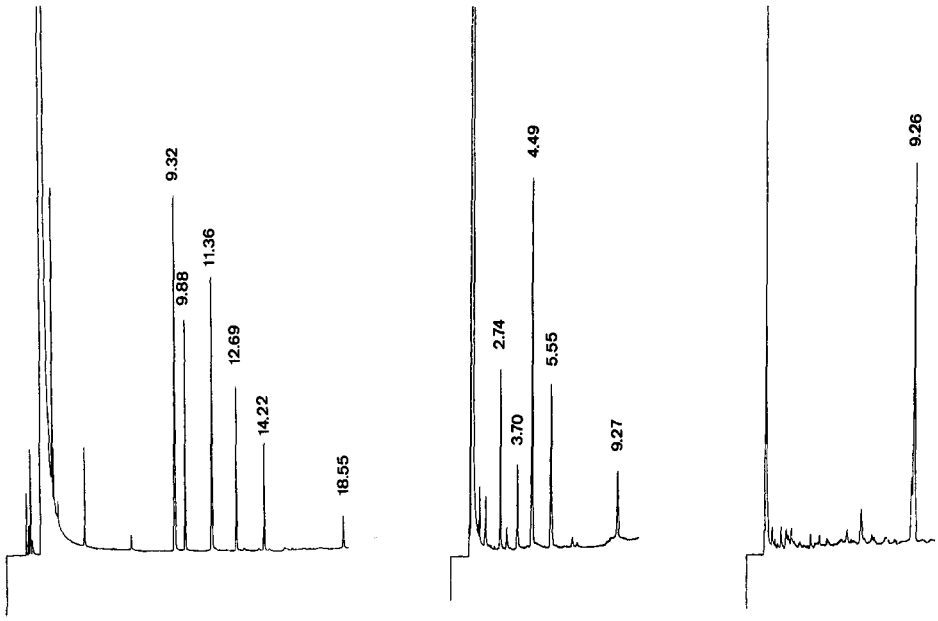

Fig. 1. Determination of chlorophenols in spiked water samples by GC with FID. Chromatographic conditions are given in the text. Concentrations of chlorophenols are given in the first column of Table I. Injection volume, 0.2 μl. Retention times (min): *o*-chlorophenol, 9.32; *p*-chlorophenol, 9.88; 3,4-dichlorophenol, 12.69; 2,4,5-trichlorophenol, 14.42; pentachlorophenol, 18.55; *p*-bromophenol (I.S.), 11.36 (all compounds as acetates).

Fig. 2. Determination of chlorophenol acetates in a 100% recovery standard by GC with ECD. Chromatographic conditions are given in the text. Concentrations of chlorophenols are given in the second column of Table II. Injection volume, 0.5 μl. Retention times (min): *o*-chlorophenol, 2.74; 3,4-dichlorophenol, 4.49; 2,4,5-trichlorophenol, 5.55; pentachlorophenol, 9.27; *p*-bromophenol (I.S.), 3.7 (all compounds as acetates).

Fig. 3. Analysis of a tap water sample spiked with 1.5 μg/l of pentachlorophenol by GC with ECD. Retention time of pentachlorophenol acetate: 9.26 min.

The applicability of the method is demonstrated in Fig. 3; tap water spiked with 1.5 μg/l of pentachlorophenol was analysed by the described procedure. The time necessary for one analysis is less than 25 min.

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Note

Ultra-thin electrophoretic gel densitometry using photo-thermal deflection spectroscopy

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(First received August 18th, 1988; revised manuscript received March 17th, 1989)

The ability to determine low quantities of absorbing species with high spatial resolution has stimulted increasing interest in photo-thermal lensing techniques, since these were first described by Gordon *et al.*[1]. Photo-thermal lensing occurs as energy from a light source with a Gaussian intensity profile, such as a laser, causes local heating in an absorbing medium about the beam axis. A radial temperature distribution following the intensity profile is formed, resulting in a refractive index gradient which acts as a lens. The strength of the lens is dependent on a number of factors: the power of the (pump) beam, the amount and the extinction coefficient of the absorbing species, together with the properties of the medium in which the lens is formed, the change in its refractive index with temperature (dn/dT), its thermal conductivity and its specific heat capacity. A second (or probe) beam is used to measure the strength of the lens. As it passes through the lens the probe beam is defocussed if it is colinear, or if not, it is deflected, both resulting in a drop in intensity at the initial position of the beam centre. This technique has been applied to ultra-sensitive absorbance measurements in a number of applications, including liquid chromatography[2–4], thin-layer chromatography[5] and gel electrophoresis[6,7]. Morris and Peck[8] have reviewed concisely the use of photo-thermal lensing techniques to chemical analysis.

We report on the use of photo-thermal deflection spectroscopy as a sensitive measure of proteins stained with Coomassie Brilliant Blue R350 after separation using ultra-thin gel electrophoresis. An orthogonally crossed beam configuration was used to measure the lens formed as the gels were scanned. We have demonstrated a limit of detection of 3.5 ng of protein per band, which is a four-fold improvement on the laser scanning densitometers currently used.

EXPERIMENTAL

Ultra-thin polyacrylamide gel electrophoresis (PAGE) was carried out using the Pharmacia (Uppsala, Sweden) "PhastSystem". Ready poured PhastGel sodium dodecyl sulphate (SDS) 10–15% (w/v) polyacrylamide gradient gels (43 × 50 × 0.45 mm) were used. Low relative molecular mass (M_r) markers (Pharmacia) were treated with 2.5% (w/v) SDS and 5% (v/v) 2-mercaptoethanol before use. The stock solution (1 ml) contained 0.32 mg of phosphorylase *b* (M_r 94 000), 0.42 mg of bovine serum

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albumin (M_r 67 000), 0.73 mg of ovalbumin (M_r 43 000), 0.42 mg of carbonic anhydrase (M_r 30 000), 0.40 mg of soybean trypsin inhibitor (M_r 20 100), 0.61 mg of lactalbumin (M_r 14 400). This was diluted serially by two and 1 μl was added to each track. The gels were electrophoresed at 250 V and 10 mA for about 30 min (60 Vh), then stained with Coomassie Brilliant Blue R350 and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. The resulting gels (Fig. 1) were dried in air at 37°C before use.

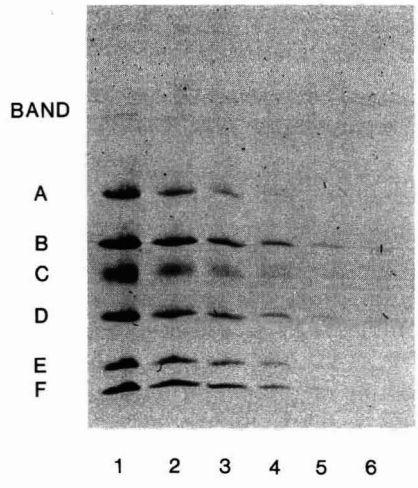

Fig. 1. Photograph of the electrophoretogram used for scanning. Lanes 1 to 6 only are shown, as the further dilutions were not visible. Bands A to F are phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lactalbumin, respectively. Lane 1 was loaded with 320 ng A, 420 ng B, 730 ng C, 420 ng D, 400 ng E, and 610 ng F. Lane 2 and subsequent lanes were produced by two-fold serial dilutions of this sample.

The gels were scanned using a conventional laser densitometer (Model Chromoscan 3; Joyce-Loebl, Gateshead, U.K.) as a direct comparison for the reported method.

The photo-thermal densitometer was configured as shown in Fig. 2. The lens was formed and detected as described by Boccara *et al.*[9]. A 10-mW helium–neon (pump) laser (Model 106-1; Spectra-Physics, St. Albans, U.K.) was focussed using a 15-cm lens to a beam waist of approximately 100 μm. An argon-ion (probe) laser (Model 2020-03, Spectra-Physics) was focussed with a similar lens to intercept the helium–neon beam waist at its own focal point. The argon-ion laser was used for convenience; a low-power helium–neon laser, or a small portion split from the pump beam, would be equally suitable as a probe beam. The gel was submerged in tetrachloromethane and mounted on a three-dimensional translation stage for accurate alignment. The stage was motorised in one dimension and gels were scanned at a rate of 2.5 mm/min. The photodiode detector was placed close (2 cm) to the gel sample and the probe beam was centred on a 25-μm pinhole placed immediately in front of the photodiode.

The pump beam was modulated at 8.25 Hz using a light chopper (Model 9479;

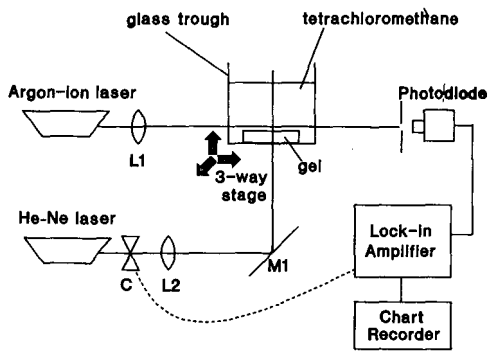

Fig. 2. Schematic diagram of the gel scanning apparatus. L1 and L2 are lenses of focal length 15 cm. M1 is a front silvered mirror and C is the light chopper, providing a reference for the lock-in amplifier.

EG+G Instruments, Bracknell, U.K.) and this provided the reference signal for the lock-in amplifier (Model 5208, EG+G Instruments) which was operated with a 1-s time constant. The signal from the lock-in amplifier was recorded directly with a chart recorder.

RESULTS AND DISCUSSION

Ultra-thin gel electrophoresis is rapidly gaining in popularity because it allows separation of proteins with a relatively high resolution, but requires only a fraction of the time to process compared with standard gels. Typically about 90 min are needed to load, run, fix, stain and destain such a gel. Ultra-thin gel electrophoresis is particularly suitable for photo-thermal measurement techniques for several reasons. It results in sharper bands than can be achieved using a standard size gel, so the protein is more concentrated and will stain more strongly, producing a stronger thermal lens. Further concentration of stained protein and corresponding increase in sensitivity, is achieved by drying the gel. The thinness of the dried gel permits rapid heat flow to the bathing solvent maximising lens formation, whereas undried, swollen gels contain water which acts as a heat sink reducing analytical sensitivity. (Swollen, undried gels also exhibit greater variation in depth reducing reproducibility in analysis). The narrow beam waists of the lasers allow high spatial resolution so the gel tracks can be narrower and protein bands can be closer than those of standard gels.

The results of scanning the gel with both the conventional laser scanner and photo-thermal spectroscopy are shown in Fig. 3. Whilst the limit of sensitivity with the conventional scanner is 14 ng of protein per band (lane 6, Fig. 3b), photo-thermal spectroscopy can detect levels of 3.5 ng of protein per band (half the signal shown in lane 7, Fig. 3a; mean signal-to-noise ratio 2:1). This compares with previous results[7] and was achieved using only a fifth of the pump laser power (which directly determines the strength of the thermal lens) and without the use of computer-aided noise reduction procedures, suggesting that the limit of detection can be reduced still further. The effects of thermal movements in the air, which caused the beams to fluctuate non-specifically in a previously described system[7] were reduced by immersing the gel in solvent and using a short pathlength between sample and diode. The requirement

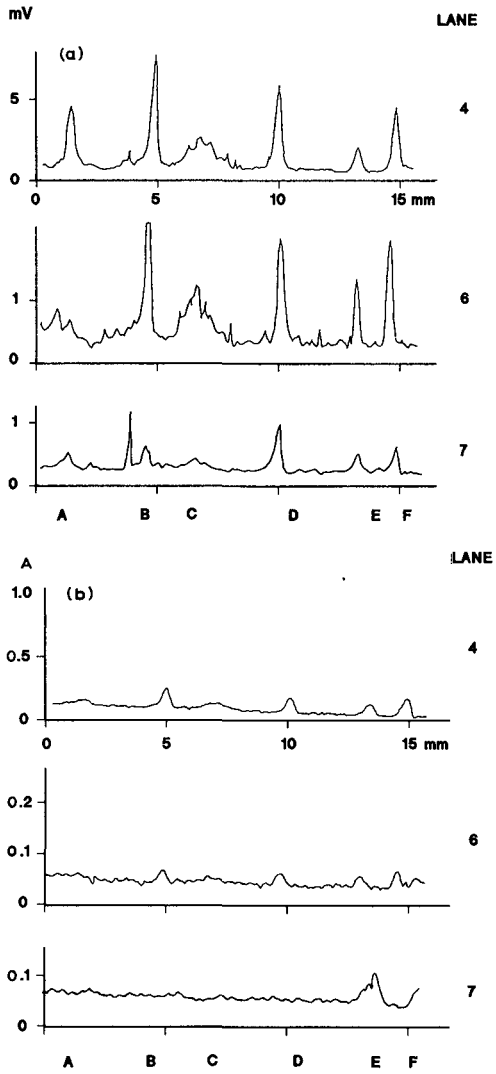

Fig. 3. Scans of the gel produced by (a) photo-thermal deflection. The lock-in amplifier was operated in high-stability mode and at low frequency, with 1 s time constant and 20 mV range. The chopper was set at 7.0 Hz. The chart recorder was operated with a paper speed of 10 cm/min and 5 V range. The scan speed was 2.5 cm/min. (b) Laser densitometry. The densitometer had 0.5 A range and an aperture width of 0.1 mm. Bands are labelled A to F as described in Fig. 1.

for a long pathlength to magnify the signal which other workers have employed[10] was overcome using a small pinhole in front of the detector.

A lower detection limit was most likely achieved by photo-thermal spectroscopy because of the lower background signal in this system. The probe laser did not pass through the gel and was therefore unaffected by inhomogeneities in the gel such as dust, air bubbles, scratches or non-specific absorption. Furthermore the gel was im-

mersed in tetrachloromethane which enhances the photo-thermal deflection by a factor of 40 compared with water[11].

Silver-stained gels have also been scanned, but in these cases photothermal spectroscopy had no advantage over conventional laser densitometry, because of the higher background encountered with this staining technique. Modification of the staining procedure produced a clear background, but at the expense of sensitivity of protein staining.

Photo-thermal spectroscopy, in common with other densitometry techniques, gives a direct measure of the amount of stain associated with a protein, but does not usually give an accurately quantitative measure of protein because of variation on the degree to which different proteins will stain. Similarly, local protein concentration variations exist within the band. We are now determining the differential staining for each protein and variations in protein distribution within a band to enable more accurate quantitation of protein concentration by integration of peak areas.

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Note

Separation of diastereomers of protected dipeptides by normal-phase high-performance liquid chromatography

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The study of racemization in peptide synthesis has long been of concern to peptide chemists. Usually the extent of racemization was determined by analysing a pair of diastereomeric peptides produced during peptide synthesis by a conventional chemical method. Several methods have been developed for determining the ratio of diastereomers quantitatively, including ^{1}H NMR spectroscopy[1,2] and high-performance liquid chromatography (HPLC)[3,4]. Diastereomeric protected dipeptides can be separated by both reversed- and normal-phase HPLC, whereas diastereomic free peptides are separated by ion-exchange chromatography and determined via the ninhydrin reaction[5,6]. Owing to the greater complication and the possibility of adverse effects on the ratio of diastereomers when one or two deprotecting steps are applied, the direct analysis of protected peptides provides a means of significantly simplifying racemization studies. Systematic studies of the reversed-phase HPLC separation of protected peptides have been reported[3,4,7,8], but there are only a few examples of normal-phase separations[9–12]. In this work we used X–D, L–AA_1–L–AA_2–OBzl as model compounds, where Bzl is benzyl and X could be formyl (For-), acetyl (Ac-), *tert.*-butyloxycarbonyl (Boc-), benzoyl (Bz-) and benzyloxycarbonyl (Z-) groups and AA_1 and AA_2 are Ala and Phe, to study systematically the separation of these diastereomeric pairs by normal-phase HPLC.

EXPERIMENTAL

L-Amino acids were purchased from Kyowa Fermentation (Tokyo, Japan) and D-amino acids from Sigma (U.S.A.). All solvents were obtained from Alps Chemical (Taiwan). Thin-layer chromatography was performed on silica gel GF_{254} (type 60) from E. Merck (F.R.G.). Ninhydrin reagent was used to locate the carboxyl- and N-protected amino acids and synthetic dipeptides were detected by the chlorine–tolidine method[13]. N- or carboxyl-protected amino acids were prepared by conventional methods and protected dipeptides were synthesized by the dicyclohexylcarbodiimidecoupling method.

An HPLC system from Waters Assoc. (Milford, MA, U.S.A.) was used for the analytical separations, consisting of one M6000A solvent-delivery unit and a U6K universal liquid chromatograph injector, coupled to an M450 variable-wavelength UV spectrophotometer and an Omniscribe two-channel chart recorder (Houston In-

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struments, Austin, TX, U.S.A.). Integration was performed electronically with an SP4100 computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.). Diastereomeric dipeptides were separated on a silica gel column (25 cm × 4 cm I.D.) (E. Merck) using 2-propanol (IPA) in *n*-hexane or chloroform as the mobile phase and detected at UV 254 nm.

TABLE I

SEPARATION OF PROTECTED DIASTEREOMERIC PEPTIDES

IPA = 2-Propanol; Hex = *n*-hexane; $CHCl_3$ = chloroform.

Compound		*k'*	*Separation factor (α)*	*Eluent*
Bz–Phe–Phe–OBzl	L–L	2.53	1.58	IPA–Hex(0.9:100)
	D–L	4.00		
Ac–Phe–Phe–OBzl	L–L	3.33	1.40	IPA–Hex(3.6:100)
	D–L	4.66		
For–Phe–Phe–OBzl	L–L	3.66	1.36	IPA–Hex(3.6:100)
	D–L	5.00		
Z–Phe–Phe–OBzl	L–L	4.73	1.27	IPA–Hex(0.6:100)
	D–L	6.00		
Boc–Phe–Phe–OBzl	L–L	4.00	1.25	IPA–Hex(0.5:100)
	D–L	5.00		
Bz–Ala–Phe–OBzl	L–L	4.73	1.23	IPA–Hex(1.5:100)
	D–L	5.80		
Ac–Ala–Phe–OBzl	L–L	3.75	1.15	IPA–$CHCl_3$(3.0:100)
	D–L	3.25		
For–Ala–Phe–OBzl	L–L	2.60	1.21	IPA–$CHCl_3$(4.0:100)
	D–L	2.15		
Z–Ala–Phe–OBzl	L–L	7.13	1.12	IPA–Hex(1.6:100)
	D–L	8.00		
Boc–Ala–Phe–OBzl	L–L	6.00	1.03	IPA–Hex(1.4:100)
	D–L	6.18		
Bz–Phe–Ala–OBzl	L–L	4.27	1.20	IPA–Hex(1.2:100)
	D–L	3.53		
Ac–Phe–Ala–OBzl	L–L	6.33	1.16	IPA–Hex(4.0:100)
	D–L	5.47		
For–Phe–Ala–OBzl	L–L	2.50	1.50	IPA–$CHCl_3$(3.0:100)
	D–L	1.65		
Z–Phe–Ala–OBzl	L–L	5.13	1.26	IPA–Hex(0.7:100)
	D–L	4.06		
Boc–Phe–Ala–OBzl	L–L	7.67	1.31	IPA–Hex(0.6:100)
	D–L	5.87		
Bz–Ala–Ala–OBzl	L–L	4.20	1.50	IPA–Hex(2.0:100)
	D–L	6.33		
Ac–Ala–Ala–OBzl	L–L	2.60	1.13	IPA–$CHCl_3$(7.0:100)
	D–L	2.30		
For–Ala–Ala–OBzl	L–L	3.75	1.19	IPA–$CHCl_3$(7.0:100)
	D–L	3.15		
Z–Ala–Ala–OBzl	L–L	5.00	1.40	IPA–Hex(3.6:100)
	D–L	7.00		
Boc–Ala–Ala–OBzl	L–L	4.80	1.18	IPA–Hex(1.7:100)
	D–L	5.67		

RESULTS AND DISCUSSION

Recently, Benoiton *et al.*[14] used reversed-phase HPLC to separate more than 50 diastereomeric N-protected di-, tri- and tetrapeptide acids and esters and the results were very successful in three quarters of the cases. However, with Phe–Phe and Ala–Phe dipeptides, no separation could be observed and most pairs of diastereomeric peptides were separated with long retention times in the reversed-phase mode, usually more than 30 min and in some instances even longer than 90 min. Therefore, we chose Phe and Ala dipeptides as model peptides for the study of normal phase HPLC.

The data for the separation factors (α) and capacity factors (k') listed in Table I indicate that all pairs of diastereomeric dipeptides except Boc–L, D-Ala–L-Phe–OBzl can be well separated and with short retention times (less than 20 min) by normal phase HPLC (Fig. 1).

This excellent separation method can be used to study racemization in peptide synthesis. Previously reported elution orders of D–L and L–L protected peptides indicated that L–L dipeptides seem to have shorter retention times than D–L dipeptides in reversed-phase HPLC[3,4,7,8]. Our results for the normal-phase HPLC separation of

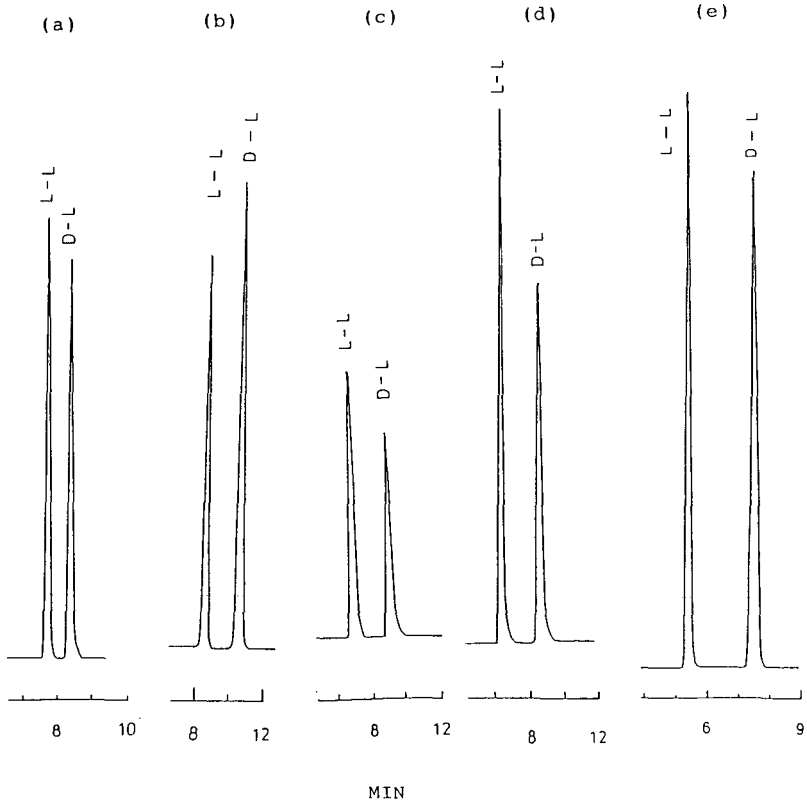

Fig. 1. HPLC profiles of diastereomeric protected dipeptides X–Phe–Phe–OBzl. (a) Boc–D,L-Phe–L-Phe–OBzl; (b) Z–D,L-Phe–L-Phe–OBzl; (c) For–D,L-Phe–L-Phe–OBzl; (d) Ac–D,L-Phe–L-Phe–OBzl; (e) Bz–D,L-Phe–L-Phe–OBzl. Elution conditions as described under Experimental and in Table I.

X–Phe–Phe–OBzl indicated that L–L species always eluted faster than D–L and for X–Phe–Ala–BOzl D–L species have shorter retention times than L–L, regardless of the different protecting groups on the N-terminus or the components of mobile phase. For the other two dipeptides, X–Ala–Ala–OBzl and X–Ala–Phe–OBzl, the N-protecting groups (X) have a critical influence on the elution order: those L–L dipeptides containing bulky N-protecting groups such as Bz, Boc and Z are eluted faster than D–L, whereas L–L dipeptides containing small N-protecting groups such as Ac and For are eluted more slowly than L–L. It is believed that the elution order of diastereomeric protected peptides is influenced by protecting groups, amino acid residues and eluents. This study has demonstrated that in some instances normal-phase HPLC can provide a better means than the reversed-phase mode for separating diastereomeric protected peptides.

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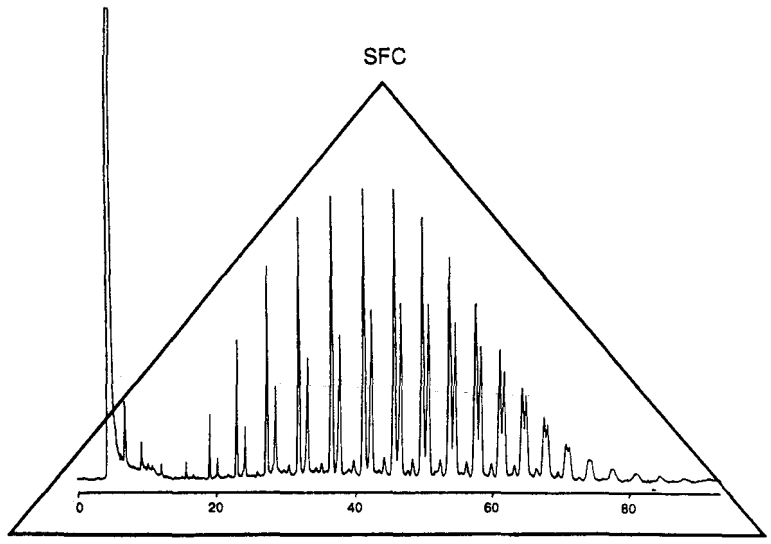
CHROM. 21 261

Book Review

Microbore column chromatography (*Chromatographic Science Series*, Vol. 45), edited by F. J. Yang, Marcel Dekker, New York, Basle, 1989, VIII + 405 pp., price US$ 99.75 (U.S.A. and Canada), US$ 119.15 (rest of world), ISBN 0-8247-7989-4.

The reviewer of a recent book [*J. Chromatogr.*, 455 (1988) 444] deplored the fact that three books on microcolumn high-performance liquid chromatography (HPLC) were already available to the reader and that, unfortunately, they showed considerable overlapping. We now have a fourth volume on this topic, but it does not overlap too much with the other three, and in fact includes several chapters on capillary supercritical fluid chromatography (SFC) and its coupling with mass spectrometers.

One point that worries the reviewer, however, is the picture shown on the cover of the book, which is repeated in the first chapter, entitled "Microbore column chromatography: a unified approach to chromatography", by the Editor of the volume. In this chapter the reader is given to understand that the author is of the opinion that there are only three modes of chromatography, namely gas chromatography (GC), SFC and LC. Even if limited only to capillary methods, one is tempted to question the



Chromatography triangle of capillary GC, capillary SFC, and micro-HPLC. The figure depicted the high resolution capability of the microbore column chromatography. A unified approach to chromatography for the application sample domain of GC, SFC, and HPLC. Chromatogram of the separation of a polyglycol with an average molecular weight of 1800 (chromatogram courtesy of B. E. Richter, Lee Scientific). Reprinted by courtesy of Marcel Dekker, Inc.

non-inclusion of capillary micellar electrokinetic chromatography and capillary electrophoresis. The reviewer believes that a generalization is attempted here on very shaky grounds.

Returning to the above-mentioned figure (Fig. 1 on p. 2), which is reproduced here, one asks oneself why GC is on the left, SFC on top and HPLC on the right, and why a chromatogram is placed in the centre with no explanation of the reason for its position in this "magic" triangle. This chromatogram is shown again on p. 267 and was obtained in SFC. Such symbolism without adequate explanation is disconcerting in a scientific work.

Journal of Chromatography, 472 (1989) 342–343
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CHROM. 21 437

Book Review

Gas and liquid chromatography in analytical chemistry, by R. M. Smith, Wiley, Chichester, 1988, XIV + 402 pp., price US$ 130.00, ISBN 0-471-90980-7.

This book by Dr. Roger Smith, Reader in Analytical Chemistry at Loughborough University of Technology, U.K., deals with the analytical aspects and possibilities of application of gas and liquid chromatography. It is divided into 16 chapters, followed by 3 appendices and a subject index. The first two chapters present a brief historical introduction to and basic concepts of chromatography. Chapters 3–7 summarize information on gas–liquid chromatography (GLC), its instrumentation, column technology, detection, identification and quantitation and special techniques of GLC. Chapter 8 deals briefly with liquid chromatographic systems, including ion-exchange and gel-permeation chromatography. Chapter 9 illustrates the possibilities of thin-layer chromatography. The subject of Chapters 10–14 is high-performance liquid chromatography, its instrumentation, detection, problems with columns and mobile phases, quantitative and qualitative analysis and special techniques, including derivatization, separation of chiral substances and optimization. Chapter 15 treats data handling and automation and the last chapter deals with future developments, mentioning also recent variants such as supercritical fluid chromatography, field-flow fractionation and affinity chromatography. In the appendices the sources of chromatographic information, trouble-shooting and a list of terms and definitions are presented. The book ends with a brief subject index.

Overall, this is a successful, concise survey written by an experienced lecturer. The book is intended for students and beginners to acquire comprehensive basic knowledge on important separation methods which are fundamental to present-day analytical chemistry. The book can be recommended, despite the comments made below.

The title refers to gas chromatography, but gas–solid chromatography is not mentioned at all, although gas analysis and trace analysis by gas–solid systems are important practical aspects that should have been mentioned explicitly (even the helium detector for gas analysis is not mentioned in the chapter on detectors, p. 431).

Although the book stresses applications in analytical chemistry, I consider that the measurement of physico-chemical quantities and parameters is as important an analytical application as the analysis of industrial products or processes in nature. Therefore, at least a short section in the part dealing with special techniques (pp. 160–178) should have presented the main analytical possibilities of the methods summarized, *e.g.*, in the monograph by J. R. Conder and C. L. Young, *Physico-chemical measurements by gas chromatography* (Wiley, 1979), or the new possibilities offered by N. A. Katsanos, *Flow perturbation gas chromatography* (Marcel Dekker, 1988).

Future developments of analytical separation methods are presented but

without even a paragraph dealing with trends in micellar liquid chromatography or in "electrochromatography" (liquid chromatography using an electrical field as a driving force), or with trends in other purely electrophoretic methods that utilize the theoretical knowledge and instrumental experience accumulated in gas chromatography and high-performance liquid chromatography (capillary zone electrophoresis as an example analogous to the elution mode of chromatography and isotachophoresis analogous to the displacement mode).

In a book intended especially for students, it is preferable for educational reasons not to refer only to the latest literature. I would have liked to have seen in the Introduction or elsewhere some mention of older works that deeply influenced the development of modern chromatography, *e.g.* the monographs by A. I. M. Keulemans, C. S. G. Phillips, E. Bayer, J. H. Purnell, A. Zlatkis and others. I also missed a reference to the work of J. C. Giddings, who introduced field-flow fractionation techniques (mentioned on p. 364) and has carried out extensive investigations in this field over the last 15 years. Of course, it is a matter of opinion how broad the choice of literature should be to suit the purpose of the book. Nevertheless, a reference to a thin (but very good) book by W. E. Harries and H. W. Habgood on temperature-programmed gas chromatography (dealt with on p. 61) and to a comprehensive German monograph by D. Jentsch and E. Otte on gas chromatographic detectors (p. 140) should have been made. The tandem technique of gas chromatography–mass spectrometry, which is very important, particularly for the progress of knowledge in life sciences, would have benefitted from a reference, *e.g.*, to the three-volume compendium by B. J. Gudzinowicz, W. J. Gudzinowicz and H. F. Martin, *Fundamentals of integrated gas chromatography–mass spectrometry* (Marcel Dekker, 1977), or to the latest book by F. W. Karasek and R. E. Clement, *Basic gas chromatography–mass spectrometry —Principles and techniques* (Elsevier, 1988). Similarly, the chapter on quantitative gas chromatography (p. 158) should have referred to the comprehensive monograph by G. Guiochon and G. L. Guillemin, *Quantitative gas chromatography for laboratory analysis and on-line control* (Elsevier, 1988).

In conclusion, I would like to repeat that the book is a well conceived source of relevant informaton suitable for teaching purposes and as an introduction to gas–liquid and high-performance liquid chromatography. My comments above were only intended to try to make it more complete.

Brno (Czechoslovakia) JAROSLAV JANÁK

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CHROM. 21 464

Book Review

High-performance liquid chromatography —Advances and perspectives, Vol. 5, edited by Cs. Horváth, Academic Press, San Diego, New York, London, 331 pp., price US$ 75.00, ISBN 0-12-312205-8.

The fifth volume of this excellent series contains three articles. The first, by Unger *et al.*, surveys bonded silica phases for biopolymers. They give extensive tables of commercially available phases, some 12 pages long, and a list of suppliers contains 35 addresses. Thus the reader is offered a thorough reference work listing also 300 references, of which a high proportion are from this journal.

The second review is on high-performance affinity chromatography, by Bergold, Hanggi, Muller and Carr. There is, of course, much in the introduction which concerns affinity chromatography in general and not only the high-performance mode. In Part II the individual studies are surveyed, including the literature up to 1987. Part III, dealing with fundamental aspects, is again more general, for example the plate height equation on page 139 is surely not limited to high-performance affinity, nor is the split-peak phenomenon specific for affinity (page 155). Part VII, entitled "The Future" starts with the phrase: "We believe that the future for HPAC is very bright", and one can only agree with the authors.

The final chapter, by Frenz and Horváth, deals with high-performance displacement chromatography, a field in which the authors have done pioneering work. It is extremely well written, especially the historical aspects, and is a must for everyone who is doing preparative chromatography, especially in biotechnology. There should be more reviews by the Editor of the series!

Journal of Chromatography, 472 (1989) 345
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 491

Book Review

Analytical methods for pesticides and plant growth regulators, Vol. XVI, Specific applications, edited by J. Sherma, Academic Press, New York, London, Sydney, 1988, 268 pp., price US$ 65.00, ISBN 0-12-784316-7.

This volume is the first to be published after the death of Dr. G. Zweig, who began this series in 1963 and which is now edited by one of his former collaborators, Dr. J. Sherma. It discusses analytical methods for two insecticides and five herbicides, which have not been discussed before in this series, and also the determination of fenvalerate is updated from Vol. XIII. There are also major chapters on anticoagulant rodenticides and fumigants and an updated coverage (previously in Vol. XIII) of pyrethroids.

Most compounds are treated in a monographic way, as is usual in pharmacopoeias, with the structural formula, alternative names, source of analytical standard biological properties, history, physical and chemical properties and formulations given under separate headings, before the actual methods are mentioned. These usually start with a short review and the recommended method is given in full detail, for both formulations and residue analysis.

A number of the compounds so treated will also be of interest in other environmental fields, *e.g.*, hydrocyanic acid, methyl bromide, phosphine, to mention only a few.

The book is attractively produced and only minor printing errors were noted.

Journal of Chromatography, 472 (1989) 346
lsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 446

Journal Review

The Journal of Microcolumn Separations, Vol. 1, No. 1 (1989), Aster Publ. Corp., 859 Willamette Street, P.O. Box 10460, Eugene, OR 97440-1046, U.S.A.; Editor: M. L. Lee, Associate Editors: J. W. Jorgenson, K. Jinno, B. O. Josefsson and M. Novotny; publication: bimonthly; subscription price US$ 175.00/year (U.S.A.), US$ 215.00 (elsewhere).

There has been a proliferation of new chromatography journals lately, such as one on preparative chromatography, another on planar chromatography and one on biomedical applications. So, faced with the task of reviewing another newcomer, one reflects on the pros and cons of this plethora. Actually, any group of research workers can start a new journal, provided that their and their friends' and associates' scientific output is sufficient to fill the initial issues. However, its success from the point of view of the publisher and the scientific community will depend on the buying public. If too few copies find their way on to the market, then the publishers, authors and readers will be dissatisfied, the last mentioned because a portion of the literature will be practically unavailable to many research workers. Hence as long as a new journal is reasonably successful, it should be welcome.

To be successful, however, a journal must also offer good service. If too narrow a topic is chosen, the new journal will find itself at a disadvantage. In the case of microcolumn separations, one really wonders whether the topic will present sufficient facets to attract a large public. Success depends to some extent on the service offered to authors and readers. In the case of a first issue, it is still too early to say whether publication will be sufficiently rapid to satisfy the users.

The American Chemical Society's ethical guidelines for journals give a standard against which this first issue may be examined. Taking as an example the paper by Y. Walbroehl and J. W. Jorgenson on page 41, we find that the scientific background is not well presented. First, the Tiselius apparatus that was the precision method of the 1950s for measuring diffusion is not mentioned, and the new results presented are hence not critically compared with the Tiselius method. Also, the work of several others on diffusion under similar conditions, but not on a micro-scale, is not cited, *e.g.*, the work of Q. P. Peniston, H. D. Agar and S. L. McCarthy, *Anal. Chem.*, 23 (1951) 994. The experimental part is sketchy; although the diffusion equations shown involve temperature, the actual temperature at which the authors worked is not given. Hence one is forced to the conclusion that the editing of this first issue has not adhered over-meticulously to the guidelines for scientific journals.

Finally, one could query the title, *The Journal of Microcolumn Separations*; this does not mention chromatography or electrophoresis. Might this not give rise to confusion amongst potential readers who are not working in separation methods? However, the next few issues will certainly show the merits of this new venture.

PUBLICATION SCHEDULE FOR 1989

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	J	J	A	S	
Journal of Chromatography	461 462 463/1	463/2 464/1	464/2 465/1 465/2	466 467/1 467/2	468 469 470/1 470/2	471 472/1 472/2 473/1	473/2 474/1 474/2 475	476 477/1 477/2		The publication schedule for further issues will be published later
Bibliography Section		486/1		486/2		486/3		486/4		
Biomedical Applications	487/1	487/2	488/1 488/2	489/1 489/2	490/1 490/2	491/1	491/2	492 493/1	493/2	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 445, pp. 453–456. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

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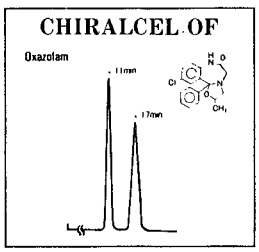
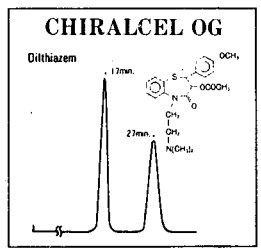
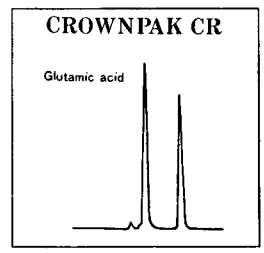
Carbinoxamine	1.39	OD
Carteolol	1.86	OD
Chlophedianol	2.82	OJ
Chlormezanone	1.47	OJ
Cyclopentolate	2.47	OJ
Diltiazem	1.46	OD
	2.36	OF
	1.75	OG
Disopyramide	2.46	OF
Ethiazide	1.54	OF
Ethotoin	1.40	OJ
Fenoprofen	1.35	OJ
Glutethimide	2.48	OJ

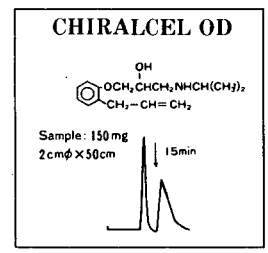
SUBSTANCE	α	column
Gauifenesin	2.40	OD
Hexobarbital	1.7	CA-1
Homatropine	3.13	OD
Hydroxyzine	1.17	OD
Indapamide	1.58	OJ
Ketamine	complete resolution	CA-1
Ketoprofen	1.46	OJ
Mephobarbital	5.9	OJ
	2.3	CA-1
Methaqualone	2.8	CA-1
	7.3	OJ
Methsuximide	2.68	OJ
Metoprolol	complete resolution	OD
Mianserin	1.75	OJ
Nilvadipine	complete resolution	OT

SUBSTANCE	α	column
Oxapadol	complete resolution	CA-1
Oxazepan	4.36	OC
Oxazolam	1.67	OF
Oxprenolol	6.03	OD
Perisoxal	1.33	OF
	1.27	OD
Pindolol	5.07	OD
Piprozolin	1.7	CA-1
Praziquantal	complete resolution	CA-1
Propranolol	2.29	OD
Rolipram	complete resolution	CA-1
Sulconazole	1.68	OJ
Suprofen	1.6	OJ
Trimebutine	1.81	OJ
Warfarin	1.96	OC









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