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Determination of Beta-Blockers in Biological Material

edited by **V. Marko,** *Institute of Experimental Pharmacology, Centre of Physiological Sciences, Slovak Academy of Sciences, Bratislava, Czechoslovakia*

(Techniques and Instrumentation in Analytical Chemistry, 4C)

This is the third volume of a sub-series entitled *Evaluation of Analytical Methods in Biological Systems*. (The first two were *Analysis of Biogenic Amines* edited by G.B. Baker and R.T. Coutts and *Hazardous Metals in Human Toxicology* edited by A. Vercruysse). This new volume addresses beta-blockers - an area of research for which a Nobel Prize in Medicine was awarded in 1988. It provides an up-to-date and comprehensive coverage of the theory and practice of the determination of beta-blockers in biological material. Two main fields of research are dealt with in this book: analytical chemistry and pharmacology, and, as it deals with drugs used in clinical practice, it is also related to a third area: therapy. Thus, it offers relevant information to workers in all three fields.

Some 50 beta-blockers and nine methods of analysis are discussed. The methods are divided into three groups: optical, chromatographic, and saturation methods. In addition to the analytical methods themselves, sample handling problems are also covered in detail, as is the information content of the analytical results obtained. Special chapters are directed to those working in pharmacology and pharmacokinetics. Finally, as recent evidence points to the increased importance of distinguishing optical isomers of drugs, a chapter on the determination of optical isomers of beta-blockers in biological material is also included. An extensive subject index and two supplements giving retention indices and structures of beta-blockers complete the book.

This is the first book to treat beta-blockers from the point of view of their determination and to discuss in detail the use of analytical methods for beta-blockers. It will thus appeal to a wide-ranging readership.

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CHEMISTRY FOR PROTECTION OF THE ENVIRONMENT 1987

Proceedings of the Sixth International Conference, Torino, Italy, 15-18 September 1987

edited by **L. Pawlowski,** *Technical University of Lublin, Poland,* **E. Mentasti,** *University of Torino, Italy,* **W.J. Lacy,** *Alexandria, VA, USA,* **C. Sarzanini,** *University of Torino, Italy*

(Studies in Environmental Science, 34)

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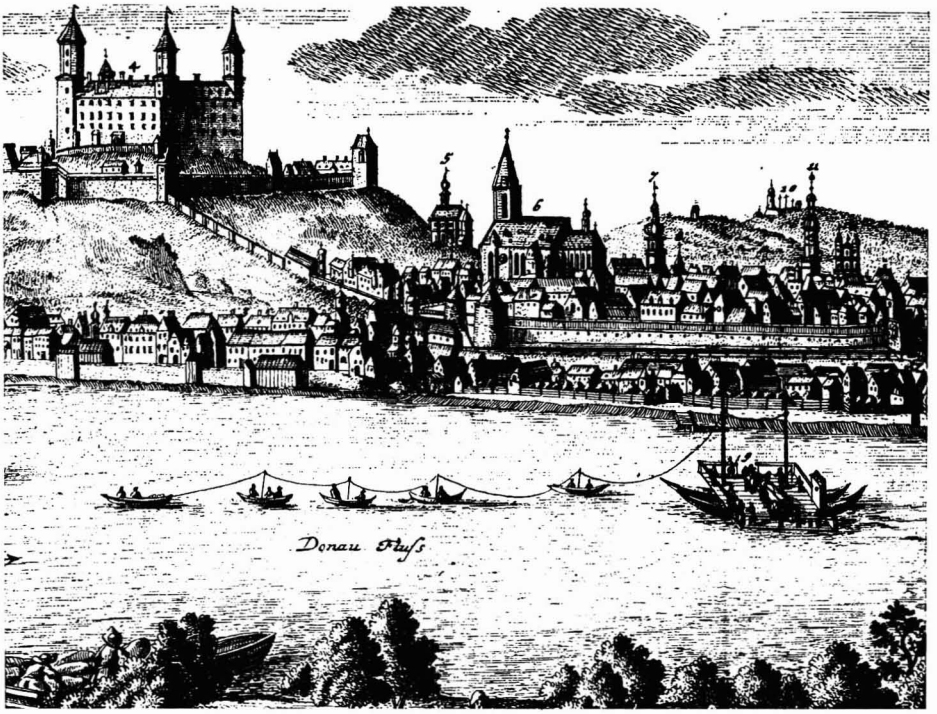

8TH INTERNATIONAL SYMPOSIUM ADVANCES AND APPLICATION OF CHROMATOGRAPHY IN INDUSTRY

Bratislava (Czechoslovakia), July 2–7, 1989

Guest Editors

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(Brno, Czechoslovakia)

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(Bratislava, Czechoslovakia)

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In articles with more than one author, the name of the author to whom correspondence should be addressed is indicated in the article heading by a 6-pointed asterisk (*)

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PREFACE

On July 2–7, 1989, the *8th International Symposium on Advances and Applications of Chromatography in Industry* took place in Bratislava, Czechoslovakia. This symposium followed previous ones held in 1967, 1971, 1973, 1977, 1980, 1983 and 1987 and was organized by local scientific societies and academic and industrial chemical bodies. About 300 participants from 21 countries took part.

The scientific programme included 10 Plenary Lectures (U. A. Th. Brinkman, C. A. Cramers, M. B. Evans, A. F. Fell, J. F. K. Huber, M. Martin, M. Novotny, Y. Okamoto, H. Poppe and P. Sandra), 44 lectures and 170 posters. In panel sessions, the theory and practice of liquid, gas and supercritical fluid chromatography, electromigration and chromatography-related methods were discussed. Many contributors were oriented towards analytical applications in industry, biotechnology, biochemistry and clinical chemistry; some of them stressed problems in trace and environmental analyses.

An exhibition of chromatographic and electrophoretic instrumentation and materials was organized within the symposium with the participation of 14 exhibitors.

The cultural programme provided traditional offerings with concerts by Slovak and foreign musicians and sight-seeing tours of Bratislava and its surroundings. Evening social events allowed useful personal contacts and exchange of opinions in a convivial atmosphere.

Brno (Czechoslovakia) JAROSLAV JANÁK

Journal of Chromatography, 509 (1990) 3–14
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Structure–retention correlations of isomeric alkylphenols in gas–liquid chromatography

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ABSTRACT

Correlations of the chromatographic retention of alkylphenols with their boiling points, molecular refraction and ionization constants were studied. The correlations were obeyed better for the alkylphenol groups which differ in the degree of shielding of the hydroxyl group. The dependences are approximated most exactly for alkylphenols with substituents at positions 2 and 6 by a four-factor equation and for the other phenols by a eight-factor equation which include the Van der Waals volume and connectivity indices of the first five orders as independent variables.

INTRODUCTION

Correlations of chromatographic retention with the physico-chemical and structural characteristics of substances are of significance for the identification of components of complex mixtures. A comprehensive analysis of the potential of different structure–chromatographic retention relationships, calculation schemes and their use in chromatographic investigations has been given in several reviews[1–6].

Studies of the structure–retention relationship are based on the principle of additivity of free energies of intermolecular interactions of the substances with the stationary phase, which are determined according to the dependence

$$\Delta G^0 = -RT \ln K \tag{1}$$

where K is the chromatographic distribution coefficient.

The widely used Kováts retention indices[7] can be expressed in units of free energy of sorption of n-alkanes:

$$I = 100 \cdot \frac{\Delta G^0 - \Delta G_n^0}{\Delta G_{n+1}^0 - \Delta G_n^0} + 100\, n \tag{2}$$

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where n is the number of carbon atoms in the n-alkane molecule.

The additive scheme suggested by Berezkin[8] for calculating the characteristics of retention by structural increments of the compound assumes additivity of retention indices[9]:

$$I = \sum_{i=1}^{n} a_i x_i + b \tag{3}$$

where x_i represents physico-chemical and structural characteristics of compound i and a_i and b are constants. Eqn. 3 may be simplified if we consider the linear correlation of the retention index to be dependent on the parameter:

$$I = ax + b \tag{4}$$

A number of workers who have studied the dependence of alkylphenol retention on different physico-chemical parameters have considered eqn. 4.

Franc[10] derived an empirical relationship between the relative retention volume and the dipole moment of isomeric alkylphenols. Karger and co-workers[11,12] showed that the Hammet equation can be used to characterize specific interaction forces between phenols and the stationary phase. Extension of the field of application of the Hammet equation permitted essential information on the mechanism of intermolecular interactions of phenols with different solvents to be obtained[13,14]. Lille[15] reported a linear dependence of retention indices and their increments on the number of carbon atoms in the side-chain of alkylphenols and induction constants of substituents. Correlation dependences of retention indices on the boiling temperature, molecular refraction and Hammet and Taft constants of alkyl substituents have been determined for a comparatively restricted number of alkylphenols[16,17]. It was shown that the linear dependence of the logarithm of the retention volume of different series of monosubstituted n-alkylphenols on the number of carbon atoms has a "break point" corresponding to n-propylphenols[18]. Hall and Kier[19] reported a direct influence of the structure of alkylphenols on their toxicity. Dmitrikov and Nabivach[20] established a correlation dependence of the relative retention times of alkylphenol in high-performance liquid chromatography on molecular connectivity indices of the first order. It has been shown that correlations for alkylphenols may be described by multi-factor equations[21].

EXPERIMENTAL

Retention indices of C_6–C_{12} alkylphenols obtained on a column packed with 5% hexaphenyl ether (HPE) on Chromatone N AW HMDS (0.16–0.20 mm) at 160°C were used[22].

Boiling temperature (t_b), molecular refraction (R_M), ionization constant (pK_a) in water and methanol and structural parameters (Van der Waals volume[23] and molecular connectivity index) were used as the variable x in eqns. 3 and 4.

The Van der Waals volumes (V_w) were calculated according to Bondi[23] by summation of the volume contributions of certain groups that form molecules of alkylphenols. For example, V_W for 3-ethyl-5-methylphenol is given by

$$V_W = V_W(\text{benzene ring}) + V_W(OH) + V_W(C_2H_5) + V_W(CH_3)$$

$$= 40.80 + 8.04 + 23.90 + 13.67 = 86.41 \text{ ml/mol}$$

The path (χ_p) and cluster ($^3\chi_c$, χ_{pc}) connectivity indices of the first five orders were calculated according to Kier and Hall[24]. Having combined the indicated indices, it is possible to obtain the total path and cluster index, *e.g.*, $^3\chi_{p+c} = {}^3\chi_p + {}^3\chi_c$, $^4\chi_{p+pc} = {}^4\chi_p + {}^4\chi_{pc}$, etc. To characterize more completely the structure of alkylphenols, the sums of connectivity indices of several orders were calculated, *e.g.*,

$$^{1-3}\chi_p = {}^1\chi + {}^2\chi + {}^3\chi_p$$

$$^{1-3}\chi_{p+c} = {}^1\chi + {}^2\chi + {}^3\chi_{p+c}$$

$$^4\chi_p = {}^1\chi + {}^2\chi + {}^3\chi_p + {}^4\chi_p$$

$$^{1-4}\chi_{p+pc} = {}^1\chi + {}^2\chi + {}^3\chi_{p+c} + {}^4\chi_{p+pc}$$

RESULTS AND DISCUSSION

Preliminary calculations of the coefficients in eqn. 4 for certain homologous series of alkylphenols with the use of connectivity indices of different orders made it possible to evaluate their significance and to choose the type of index that could provide the highest correlation. Table I shows that the sums of the path indices $^{1-3}\chi_p$, $^{1-4}\chi_p$ and $^{1-5}\chi_p$ correlate better with the retention index than the analogous path and cluster indices. Among the path indices studied, the sum of path indices of four orders, $^{1-4}\chi_p$, which simultaneously with the high level of correlation permits minimum standard deviations of the calculated retention indices to be obtained, is preferable.

The retention indices used and the physico-chemical and structural parameters of alkylphenols are given in Table II.

TABLE I

CORRELATION COEFFICIENTS AND STANDARD DEVIATIONS (i.u.) FOR EQNS. 4

Series of alkylphenols[a]	*Value of x*						
	$^1\chi$	$^{1-3}\chi_p$	$^{1-4}\chi_p$	$^{1-5}\chi_p$	$^{1-3}\chi_{p+c}$	$^{1-4}\chi_{p+pc}$	$^{1-5}\chi_{p+pc}$
3-MePh, 2,5-DiMePh, 2-Et-5-MePh,	0.9995	0.9978	0.9993	0.9992	0.9992	0.9933	0.9948
3-Me-6-*n*-PrPh	2.9	6.1	3.4	3.8	11.6	10.8	9.5
2-MePh, 2,3-DiMePh,	1.0000	0.9999	1.0000	0.9992	0.9999	0.9999	0.9997
2,3,4-TriMePh, 2,3,4,5-TetraMePh	0.9	1.9	0.4	5.1	1.8	2.8	3.1
Ph, 2-MePh, 2-EtPh, 2-*n*-PrPh	0.9987	0.9988	0.9996	0.9998	0.9968	0.9942	0.9938
	4.9	4.6	2.5	1.7	7.7	10.3	10.6
2,6-DiMePh, 2,3,6-TriMePh,	1.0000	0.9992	1.0000	0.9999	0.9994	0.9999	1.0000
2,3,5,6-TetraMePh	0.9	5.2	1.0	1.8	4.7	1.2	0.6

[a] Ph = Phenol; Me = methyl; Et = ethyl; Pr = propyl; Bu = butyl; Pe = pentyl; Hex = hexyl.

TABLE II

RETENTION INDICES AND PHYSICO-CHEMICAL CHARACTERISTICS OF PHENOLS

Compound	I_{160}^{HPE}	t_b (°C)	V_W (*ml/mol*)	$^{1-4}\chi_p$
Ph	1281	182.0	53.88	4.65402
2-MePh	1354	190.8	65.03	6.01632
3-MePh	1386	202.2	65.03	6.03592
4-MePh	1385	201.9	65.03	5.95925
2-EtPh	1430	206.0	75.26	7.27618
3-EtPh	1483	217.0	75.26	7.22427
4-EtPh	1473	218.0	75.26	7.20186
2,3-DiMePh	1495	217.1	76.18	7.50694
2,4-DiMePh	1456	211.3	76.18	7.40925
2,5-DiMePh	1453	211.5	76.18	7.33564
2,6-DiMePh	1416	200.6	76.18	7.45318
3,5-DiMePh	1489	221.7	76.18	7.45866
3,4-DiMePh	1530	226.9	76.18	7.44628
4-iso-PrPh	1527	229.1	85.48	8.65355
2-*n*-PrPh	1502	220.0	85.49	8.49285
3-*n*-PrPh	1565	233.5	85.49	8.42072
4-*n*-PrPh	1563	233.1	85.49	8.44838
2-Et-4-MePh	1523	223.3	86.41	8.62139
2-Et-5-MePh	1529	224.2	86.41	8.60257
2-Et-6-MePh	1485	213.0	86.41	8.67171
3-Et-5-MePh	1581	235.6	86.41	8.62160
4-Et-2-MePh	1539	227.0	86.41	8.60364
4-Et-3-MePh	1608	229.0	86.41	8.77046
2,3,4-TriMePh	1638	237.0	87.33	9.00709
2,3,5-TriMePh	1593	235.3	87.33	8.90280
2,3,6-TriMePh	1551	234.0	87.33	8.98058
2,4,5-TriMePh	1593	232.0	87.33	8.85882
3,4,5-TriMePh	1667	251.9	87.33	9.01393
4-*sec.*-BuPh	1612	242.1	95.71	10.09579
2-*n*-BuPh	1600	235.0	95.72	9.72462
3-*n*-BuPh	1668	250.5	95.72	9.64683
4-*n*-BuPh	1661	248.0	95.72	9.67448
2-Me-4-*n*-PrPh	1623	242.6	96.64	9.85476
2-Me-6-*n*-PrPh	1553	241.3	96.64	9.90766
3-Me-6-*n*-PrPh	1602		96.64	9.81922
4-Me-2-*n*-PrPh	1593	241.0	96.64	9.89444
2,4-DiEtPh	1602	229.0	96.64	9.90136
2,5-DiEtPh	1624	242.5	96.64	9.82030
3,4-DiEtPh	1682	252.5	96.64	10.06733
2,3,4,5-TetraMePh	1782	260.0	98.48	10.53318
2,3,4,6-TetraMePh	1690	250.0	98.48	10.60606
2,3,5,6-TetraMePh	1683	248.0	98.48	10.43240
2-Et-4,5-DiMePh	1656		97.56	10.07748
2-*n*-PePh	1700	256.2	105.95	11.02640
4-*n*-PePh	1765	266.6	105.95	10.97626
4-*tert.*-PePh	1703	264.0	105.94	11.43578
2-Et-5-*n*-PrPh	1706	257.6	106.87	11.07143
2-*n*-HexPh	1800	272.2	116.18	12.30673
4-*n*-HexPh	1871	281.3	116.18	12.26253
3-*n*-Bu-6-EtPh	1807	275.7	117.10	12.29754

TABLE III

CORRELATION COEFFICIENTS AND STANDARD DEVIATIONS (i.u.) FOR EQNS. 4

Eqn. No.	*Series of alkylphenols*	t_b		V_W		$^{1-4}\chi_p$	
		r	*s*	*r*	*s*	*r*	*s*
1	2,4-DiMePh, 2-Me-4-EtPh, 2-Me-4-*n*-PrPh	0.9999	0.4	1.0000	0.4	1.0000	0.2
2	3-MePh, 3,5-DiMePh, 2,3,5-TriMePh	0.9945	10.0	1.0000	0.3	1.0000	0.2
3	2-MePh, 2,3-DiMePh, 2,3,4-TriMePh, 2,3,4,5-TetraMePh	0.9984	10.4	1.0000	0.9	1.0000	0.3
4	2,6-DiMePh, 2-Et-6-MePh, 2-Me-6-*n*-PrPh	0.9746	15.3	1.0000	0.3	1.0000	0.6
5	3,4-DiMePh, 4-Et-3-MePh, 3,4-DiEtPh	0.8939	34.1	0.9999	1.1	1.0000	0.7
6	2-*n*-PrPh, 2-*n*-BuPh, 2-*n*-PePh, 2-*n*-HexPh	0.9980	8.0	1.0000	0.6	1.0000	0.8
7	2,6-DiMePh, 2,3,6-TriMePh, 2,3,5,6-TetraMePh	0.9747	29.8	1.0000	0.9	1.0000	1.0
8	4-EtPh, 4-Et-2-MePh, 2,4-DiEtPh	0.9956	5.9	0.9999	0.7	0.9999	1.0
9	2,6-DiMePh, 2-Et-6-MePh, 2,6-DiEtPh	0.9761	14.5	0.9998	1.3	0.9999	1.1
10	2,6-DiMePh, 2,3,6-TriMePh, 2,3,4,6-TetraMePh	0.9782	28.4	1.0000	0.9	1.0000	1.3
11	Ph, 2-MePh, 2-EtPh, 2-*n*-PrPh	0.9945	10.0	0.9997	2.4	0.9996	1.5
12	4-MePh, 2,4-DiMePh, 2-Et-4-MePh, 4-Me-2-*n*-PrPh	0.9859	14.9	0.9999	1.5	0.9996	2.6
13	3,4-DiMePh, 2,4,5-TriMePh, 2-Et-4,5-DiMePh	–	–	0.9997	1.6	0.9991	2.7
14	Ph, 4-MePh, 4-EtPh, 4-*n*-PrPh, 4-*n*-BuPh	0.9988	7.1	0.9998	2.6	0.9997	3.3
15	3-MePh, 2,5-DiMePh, 2-Et-5-MePh, 3-Me-6-*n*-PrPh	0.9986	3.8	0.9989	4.3	0.9993	3.3
16	Ph, 2,5-DiMePh, 2,5-DiEtPh	0.9999	2.7	0.9997	4.0	0.9998	3.5
17	4-EtPh, 4-*n*-PrPh, 4-*n*-BuPh, 4-*n*-PePh, 4-*n*-HexPh	0.9994	5.6	0.9995	5.2	0.9997	3.6
18	Ph, 3-MePh, 3-EtPh, 3-Et-5-MePh	0.9991	5.6	0.9998	2.3	0.9996	3.6
19	4-iso-PrPh, 4-*sec.*-BuPh, 4-*tert.*-PePh	0.9920	11.1	0.9998	1.7	0.9992	3.6
20	Ph, 3-MePh, 3-EtPh, 3-*n*-PrPh, 3-*n*-BuPh	0.9993	6.4	0.9996	4.5	0.9997	3.9
21	2-EtPh, 2-Et-5-MePh, 2,5-DiEtPh, 2-Et-5-*n*-PrPh, 2-Et-5-*n*-BuPh	0.9999	1.7	0.9997	3.9	0.9996	4.2
22	4-MePh, 3,4-DiMePh, 3,4,5-TriMePh	0.9998	2.9	0.9999	2.4	0.9995	4.4

Correlation dependences were considered for homologous series which were formed by the one-type characteristic of the successive introduction of the methylene group into the side-chain or ring of C_6–C_8 phenols. Application of eqn. 4 to such series permitted linear-regression equations of the dependence of retention indices on the molecular characteristics of alkylphenols to be obtained (Table III). The results showed that all the parameters studied correlate with the retention indices, the equations with structural characteristics V_W and $^{1-4}\chi_p$ being of greater significance and having lower standard deviations of the calculated retention indices. Thus, the correlation coefficient of equations $I = at_b + b$ is 0.8939–0.9999, whereas that of equations with V_W and $^{1-4}\chi_p$ is 0.999–1.000.

Table IV presents data on the predictive capacity of the equations obtained. Two equations producing minimum standard deviations were used to calculate the retention indices of each compound. Thus, the retention index of phenol was determined by eqns. 11 and 14, that of 2,6-dimethylphenol by eqns. 4 and 7, that of 2-ethylphenol by eqns. 11 and 21, etc. (see Table III). The results show that both structure parameters may be used for the preliminary calculation of the retention indices of alkylphenols. The error in the determination of the retention indices by $^{1-4}\chi_p$ does not exceed 4 i.u. and that of V_W 5 i.u. The use of connectivity indices is more preferable. In contrast to V_W, they differentiate isomeric compounds well.

TABLE IV

COMPARISON OF EXPERIMENTAL AND PREDICTED *I* VALUES OF ALKYLPHENOLS

Compound	I_{exp}	*Predicted from* $^{1-4}\chi_p$		*Predicted from* V_W	
		I_m	ΔI	I_m	ΔI
Ph	1281	1280.8	0.2	1280.4	0.6
2-MePh	1354	1355.7	−1.7	1355.3	−1.3
4-MePh	1385	1385.1	−0.1	1385.5	−0.5
3-MePh	1386	1384.5	1.5	1384.0	2.0
2,6-DiMePh	1416	1415.9	0.1	1416.3	−0.3
2,5-DiMePh	1453	1457.1	−4.1	1458.2	−5.2
2,4-DiMePh	1456	1457.8	−1.8	1456.4	−0.4
3,4-DiMePh	1530	1529.5	0.5	1530.0	0
2-EtPh	1430	1430.3	−0.3	1429.6	0.4
4-EtPh	1473	1473.2	−0.2	1473.4	−0.4
2-Et-6-MePh	1485	1485.2	−0.2	1484.0	1.0
2-Et-5-MePh	1529	1530.5	−1.5	1529.5	−0.4
4-Et-2-MePh	1539	1539.2	−0.2	1539.5	−0.5
2,3,6-TriMePh	1551	1550.9	0.1	1551.1	−0.1
2-*n*-PrPh	1502	1501.7	0.3	1501.3	0.7
4-*n*-PrPh	1563	1566.9	−3.9	1566.6	−3.6
2,5-DiEtPh	1624	1620.8	3.2	1621.3	2.7
4-*n*-BuPh	1661	1661.2	−0.2	1662.5	−1.5

The characteristics presented in Table V were used for studies of their influence on correlation indices according to eqn. 3.

Table VI represents multiple correlation coefficients, standard deviations and

TABLE V

PHYSICO-CHEMICAL AND STRUCTURAL CHARACTERISTICS OF ALKYLPHENOLS

Compound	R_M	*Ionization constant* (pK_a)		*Connectivity index*			
		In methanol, pK_a^m	*In water,* pK_a^w	$^1\chi$	$^2\chi$	$^3\chi_p$	$^4\chi_p$
Ph	27.992	14.20	10.02	2.1343	1.3356	0.7562	0.4280
2-MePh	32.838	14.80	10.33	2.5510	1.7865	1.1155	0.5634
4-MePh	32.874	14.55	10.27	2.5450	1.8356	1.0340	0.5448
3-MePh	32.921	14.38	10.10	2.5450	1.8613	1.0017	0.6280
2,6-DiMePh	37.797	15.27	10.63	2.9676	2.2404	1.4395	0.8057
2,5-DiMePh	38.089	14.90	10.40	2.9616	2.2899	1.3646	0.7194
2,4-DiMePh	39.843	15.05	10.60	2.9616	2.2899	1.3523	0.8054
3,5-DiMePh	38.229	14.51	10.19	2.9557	2.3459	1.2065	0.9500
2,3-DiMePh	37.885	15.09	10.53	2.9676	2.2190	1.5760	0.7443
3,4-DiMePh	38.274	14.62	10.36	2.9616	2.2686	1.4905	0.7256
2-PrPh	41.986	15.07	10.55	3.6116	2.3787	1.5110	0.9916
3-iso-PrPh	42.120	14.42	10.14	3.4877	2.7497	1.5369	0.8839
4-*n*-PrPh	42.213	14.55	10.32	3.6056	2.4165	1.4750	0.9513
2-BuPh	46.688	15.09	10.55	4.1116	2.7323	1.7913	1.0895
4-PePh	52.616	14.76	10.57	4.6056	3.1236	2.0053	1.2417

TABLE VI

REGRESSION COEFFICIENTS FOR EQNS. 3 FOR ALKYLPHENOLS

Eqn. No.	*Form of function*	*Coefficients of equations*						*r*	*s*
		a_1	a_2	a_3	a_4	a_5	b		
1	$I = f(t_b, V_W)$	4.3	2.3				383.8	0.997	8.9
2	$I = f(t_b, R_M)$	4.2	5.0				380.6	0.997	8.8
3	$I = f(t_b, pK_a^m)$	5.5	35.9				−234.0	0.998	7.4
4	$I = f(t_b, {}^3\chi_p)$	4.5	80.2				402.6	0.999	6.1
5	$I = f(t_b, V_W, {}^3\chi_p)$	4.3	0.5	68.2			409.2	0.999	5.9
6	$I = f(t_b, R_M, {}^3\chi_p)$	4.3	1.4	65.7			409.8	0.999	5.7
7	$I = f(t_b, pK_a^w, {}^3\chi_p)$	4.7	22.8	57.7			149.5	0.999	5.6
8	$I = f(t_b, pK_a^w, {}^1\chi, {}^3\chi_p)$	4.5	22.7	10.9	47.0		167.9	0.999	5.1
9	$I = f(t_b, {}^1\chi, {}^2\chi, {}^3\chi_p)$	4.5	14.3	−22.4	82.3		406.2	0.999	4.9
10	$I = f(t_b, R_M, {}^2\chi, {}^3\chi_p)$	4.4	3.4	−37.9	73.6		386.8	0.999	4.2
11	$I = f(t_b, R_M, {}^1\chi, {}^2\chi, {}^3\chi_p)$	4.4	4.5	− 7.8	−41.8	72.9	376.5	0.999	4.1
12	$I = f(t_b, R_M, V_W, {}^2\chi, {}^3\chi_p)$	4.4	4.7	− 0.7	−38.2	75.5	384.9	0.999	4.1

coefficients of multi-factor dependence equations of alkylphenol retention indices on different parameters providing the highest level of correlation with successive increases in their number.

Although specific interaction forces of alkylphenols with the stationary phase are manifested on the polar HPE, the boiling temperature reflects the greater part of such interactions and the correlation coefficient for the equation $I = at_b + b$ is 0.993.

As seen from Table VI, the simultaneous use with t_b of other characteristics (eqns. 1–4) permits the studied dependence to be improved, the introduction of the connectivity index ${}^3\chi_p$ providing the highest correlation coefficient. Further increases in the number of variables makes it possible only to decrease the standard deviation at the same correlation level of 0.999.

A positive influence of connectivity indices on the correlation level is also confirmed by the fact that all four- and five-factor equations providing minimum standard deviations in the calculation of I (e.g., eqns. 10 and 11), contain connectivity indices of different orders. At the same time, the absence of reference values for t_b, R_M, pK_a for a wide range of alkylphenols impedes considerably the prediction of their

TABLE VII

CORRELATION COEFFICIENTS AND STANDARD DEVIATIONS (i.u.) FOR EQUATION $I = f(V_W, {}^1\chi, {}^2\chi, {}^4\chi_{p+pc}, {}^5\chi_{p+pc})$ FOR DIFFERENT GROUPS OF ALKYLPHENOLS

Group of alkylphenols	*No. of compounds*	*r*	*s*
All alkylphenols	52	0.948	39.0
Group 1	20	0.997	9.8
Group 2	24	0.991	15.8
Group 3	8	0.999	3.8

TABLE VIII

REGRESSION COEFFICIENTS FOR EQNS. 3 FOR DIFFERENT GROUPS OF ALKYLPHENOLS

Group	*Eqn. No.*	*Form of function*	*Coefficients of equations*	
			a_1	a_2
Group 1	1	$I = f(V_W, {}^4\chi_p)$	7.1	115.7
	2	$I = f({}^3\chi_p, {}^5\chi_p)$	203.4	333.1
	3	$I = f(V_W, {}^1\chi, {}^3\chi_c)$	27.1	−361.7
	4	$I = f({}^3\chi_p, {}^4\chi_p, {}^5\chi_p)$	160.8	127.7
	5	$I = f(V_W, {}^1\chi, {}^2\chi, {}^5\chi_{pc})$	45.0	−585.1
	6	$I = f({}^1\chi, {}^2\chi, {}^3\chi_c, {}^4\chi_{p+pc})$	17.1	179.3
	7	$I = f(V_W, {}^1\chi, {}^2\chi, {}^4\chi_{p+pc}, {}^5\chi_{p+pc})$	50.7	−598.6
	8	$I = f({}^1\chi, {}^2\chi, {}^3\chi_c, {}^4\chi_{p+pc}, {}^5\chi_p)$	−55.3	210.4
	9	$I = f(V_W, {}^1\chi, {}^2\chi, {}^4\chi_{p+pc}, {}^5\chi_p, {}^5\chi_{pc})$	49.5	−616.8
	10	$I = f({}^1\chi, {}^2\chi, {}^3\chi_p, {}^3\chi_c, {}^4\chi_{p+pc}, {}^5\chi_p)$	−65.5	208.2
	11	$I = f(V_W, {}^1\chi, {}^2\chi, {}^4\chi_p, {}^4\chi_{pc}, {}^5\chi_p, {}^5\chi_{pc})$	49.4	−616.6
	12	$I = f({}^1\chi, {}^2\chi, {}^3\chi_p, {}^3\chi_c, {}^4\chi_{p+pc}, {}^5\chi_p, {}^5\chi_{pc})$	−64.5	204.5
	13	$I = f(V_W, {}^1\chi, {}^2\chi, {}^3\chi_p, {}^4\chi_p, {}^4\chi_{pc}, {}^5\chi_p, {}^5\chi_{pc})$	45.8	−616.8
	14	$I = f({}^1\chi, {}^2\chi, {}^3\chi_p, {}^3\chi_c, {}^4\chi_p, {}^4\chi_{pc}, {}^5\chi_p, {}^5\chi_{pc})$	−118.4	200.8
Group 2	15	$I = f(V_W, {}^4\chi_p)$	3.1	234.6
	16	$I = f({}^2\chi, {}^3\chi_p)$	127.9	198.1
	17	$I = f(V_W, {}^1\chi, {}^3\chi_p)$	9.5	−99.4
	18	$I = f({}^2\chi, {}^3\chi_p, {}^5\chi_{p+pc})$	122.5	227.7
	19	$I = f(V_W, {}^2\chi, {}^3\chi_p, {}^5\chi_p)$	3.2	134.3
	20	$I = f({}^2\chi, {}^3\chi_p, {}^4\chi_{pc}, {}^5\chi_p)$	183.2	232.7
	21	$I = f(V_W, {}^2\chi, {}^3\chi_p, {}^4\chi_{pc}, {}^5\chi_p)$	−1.1	196.3
	22	$I = f({}^1\chi, {}^2\chi, {}^3\chi_p, {}^4\chi_{p+pc}, {}^5\chi_p)$	18.9	187.8
	23	$I = f(V_W, {}^1\chi, {}^2\chi, {}^3\chi_p, {}^3\chi_c, {}^5\chi_p)$	−10.0	164.8
	24	$I = f({}^2\chi, {}^3\chi_p, {}^4\chi_p, {}^4\chi_{pc}, {}^5\chi_p, {}^5\chi_{pc})$	201.3	248.6
	25	$I = f(V_W, {}^1\chi, {}^2\chi, {}^3\chi_p, {}^4\chi_p, {}^4\chi_{pc}, {}^5\chi_p)$	−9.3	175.8
	26	$I = f({}^1\chi, {}^2\chi, {}^3\chi_p, {}^4\chi_p, {}^4\chi_{pc}, {}^5\chi_p, {}^5\chi_{pc})$	22.7	198.7
	27	$I = f(V_W, {}^1\chi, {}^2\chi, {}^3\chi_p, {}^3\chi_c, {}^4\chi_p, {}^5\chi_p, {}^5\chi_{pc})$	−14.7	282.8
	28	$I = f({}^1\chi, {}^2\chi, {}^3\chi_p, {}^3\chi_c, {}^4\chi_p, {}^4\chi_{pc}, {}^5\chi_p, {}^5\chi_{pc})$	28.9	201.3
Group 3	29	$I = f(V_W, {}^4\chi_{p+pc})$	2.7	182.8
	30	$I = f({}^1\chi, {}^4\chi_{p+pc})$	37.4	204.3
	31	$I = f(V_W, {}^3\chi_{p+c}, {}^4\chi_{p+pc})$	3.3	67.3
	32	$I = f({}^1\chi, {}^3\chi_{p+c}, {}^4\chi_{p+pc})$	51.0	71.1
	33	$I = f(V_W, {}^1\chi, {}^2\chi, {}^3\chi_c)$	69.5	−1138.0
	34	$I = f({}^1\chi, {}^2\chi, {}^3\chi_p, {}^4\chi_{p+pc})$	26.9	56.3

retention indices with the use of correlation equations. Therefore, the use in the correlation schemes of structural parameters (V_W, χ) which are determined easily from the structural formulae of the compounds, is preferred.

In order to establish correlations of retention indices with the mentioned structural parameters of alkylphenols, an attempt was made to consider all possible multi-factor regression equations of the form $I = (V_W, {}^1\chi \ldots {}^5\chi_{p+pc})$. However, the three- to five-factor equations obtained are characterized by a low correlation coefficient of 0.946–0.948 and the standard deviation reaches 39–42 i.u. The low correlation may be explained by the effect of alkyl substituents on the degree of shielding of the OH group, which in turn influences the alkylphenol retention indices.

							r	s
a_3	a_4	a_5	a_6	a_7	a_8	b		
						859.1	0.979	28.0
						1070.6	0.978	29.3
−178.3						588.1	0.992	17.6
250.5						1061.2	0.982	26.6
−201.3	−109.4					354.1	0.995	14.4
−369.7	137.9					977.2	0.989	20.4
−305.7	137.6	−308.1				240.1	0.997	9.8
−370.3	138.7	220.1				1034.2	0.991	18.2
−283.9	137.5	−186.2	−301.7			287.4	0.998	8.5
23.5	−360.0	128.5	236.2			1041.6	0.991	18.1
−282.4	142.8	138.3	188.0	−304.0		289.6	0.998	8.5
27.3	−362.1	144.2	216.2	−23.7		1042.2	0.992	18.1
−244.9	57.3	198.9	138.7	−167.8	−336.5	369.2	0.998	8.0
92.1	−305.5	265.3	145.3	175.5	−117.3	1075.4	0.992	17.5
						886.4	0.991	15.6
						893.3	0.995	11.0
176.2						783.7	0.993	13.7
−27.6						890.3	0.996	10.2
152.5	−187.7					787.1	0.997	9.0
−58.5	−201.3					845.6	0.998	7.7
255.6	72.0	−187.4				869.6	0.998	7.6
218.8	−45.6	−190.8				826.4	0.998	7.6
244.9	213.0	7.1	−179.9			961.0	0.998	7.3
−76.0	−105.1	−180.3	37.5			839.5	0.998	7.4
242.9	185.1	−36.0	21.7	−185.7		931.5	0.998	7.2
222.1	−97.4	−85.4	−188.6	40.0		822.1	0.998	7.4
289.7	165.7	3.9	−120.6	−160.7	64.7	979.5	0.998	6.8
207.3	−12.5	−100.0	−72.3	−190.2	40.5	817.9	0.998	7.3
						964.5	0.999	4.9
						1021.0	0.998	6.0
107.7						893.7	1.000	2.7
127.3						958.1	0.999	4.0
−182.3	−694.2					146.2	1.000	1.9
49.1	134.6					949.6	1.000	2.1

In order to determine the correlation dependence, it is expedient to divide the set of the alkylphenols studied into three groups differing in the degree of shielding of the hydroxyl group: group 1, phenol and alkylphenols with substituents in positions 3, 4 and 5 (unshielded alkylphenols); group 2, alkylphenols in which the shielding group is only in position 2 or 6 (partially shielded alkylphenols); and group 3, alkylphenols containing substituents in positions 2 and 6 (completely shielded alkylphenols).

In spite of the known inaccuracy of such a classification (in particular, the value of alkyl substituents is not taken into account), the manifestation of steric effects inside the enumerated groups is of the same character, which permits a higher level of correlation between the retention and structural factors. Table VII presents the results

of processing of the five-factor equation in the form $I = (V_W, {}^1\chi, {}^2\chi, {}^4\chi_{p+pc}, {}^5\chi_{p+pc})$. They show that a higher level of correlation compared with the whole set of alkylphenols is provided for each group of alkylphenols.

The path and cluster connectivity indices and also the total path and cluster indices of the first five orders and the descriptor V_W were used for the formation of the structural models of alkylphenols.

The results of statistical processing of multi-factor equations, which have the highest level of correlation in each group of alkylphenols with subsequent increase in the number of factors, are given in Table VIII. The data obtained show that an increase in the degree of shielding of the hydroxyl group of alkylphenols results in an increase in the correlation coefficient in the sequence group 1 < group 2 < group 3. In this instance the number of parameters that are required to reach approximately the same level of correlation decreases in the sequence group 1 < group 2 < group 3. Thus, if for alkylphenols of group 1 a value of $r = 0.998$ is reached with the use of the six-factor polynomial of the first power (eqn. 9) and for alkylphenols of group 2 the same value of r is obtained with the help of the four-factor equation (eqn. 20), then with group 3 compounds the analogous value of r is provided by two-factor equation (eqn. 30).

When analysing the composition and nature of the factors that constitute the equations obtained, the role of cluster indices and total path and cluster indices, which increases with the degree of shielding of hydroxyl groups, ought to be noted. Thus, for example, the fact that the total path and cluster index ${}^4\chi_{p+pc}$ is present all the equations for group 3 indicate a prevailing role of this index in models of phenol connectivity which describe the peculiarities of the retention of alkylphenols with substituents in positions 2 and 6. An analogous effect is produced by the cluster index ${}^3\chi_c$, the contribution of which to the models of molecular connectivity of group 1 is rather noticeably. In models of molecular connectivity of group 2 the contribution of the path index ${}^3\chi_p$ is rather essential.

The descriptor V_W (Table VIII) has a noticeable influence on the correlation coefficient. The additional introduction of V_W permits an increase in r (eqns. 12 and 13)

TABLE IX

INFLUENCE OF V_W AND ${}^1\chi$ ON CORRELATION COEFFICIENT OF REGRESSION EQUATIONS

Form of function	*Group of alkylphenols*		
	1	*2*	*3*
$I = f({}^1\chi, {}^2\chi)$	0.963	0.966	0.961
$I = f(V_W, {}^2\chi)$	0.978	0.967	0.963
$I = f({}^1\chi, {}^2\chi, {}^3\chi_p)$	0.973	0.996	0.980
$I = f(V_W, {}^2\chi, {}^3\chi_p)$	0.980	0.996	0.982
$I = f({}^1\chi, {}^2\chi, {}^3\chi_p, {}^4\chi_p)$	0.978	0.996	0.988
$I = f(V_W, {}^2\chi, {}^3\chi_p, {}^4\chi_p)$	0.982	0.996	0.991
$I = f({}^1\chi, {}^3\chi_{p+c})$	0.966	0.988	0.996
$I = f(V_W, {}^3\chi_{p+c})$	0.976	0.989	0.999
$I = f({}^1\chi, {}^3\chi_{p+c}, {}^4\chi_{p+pc}, {}^5\chi_{p+pc})$	0.978	0.990	0.999
$I = f(V_W, {}^3\chi_{p+c}, {}^4\chi_{p+pc}, {}^5\chi_{p+pc})$	0.984	0.990	1.000

TABLE X

EXPERIMENTAL AND PREDICTED *I* VALUES OF ALKYLPHENOLS

Group of alkylphenols	*Compound*	I_{exp}	I_{pred}	ΔI
Group 1	3-MePh	1386.0	1386.1	−0.1
	3-EtPh	1483.0	1479.2	3.8
	3,4-DiMePh	1530.0	1525.2	4.8
	4-*n*-PrPh	1563.0	1563.9	−0.9
	3-*n*-PrPh	1565.0	1571.4	−6.4
	4-*n*-BuPh	1661.0	1656.6	4.4
	4-*n*-PePh	1765.0	1769.9	−4.9
Group 2	2-MePh	1354.0	1347.6	−6.4
	2,5-DiMePh	1453.0	1450.5	2.5
	2,4-DiMePh	1456.0	1459.3	−3.3
	2-*n*-PrPh	1502.0	1499.1	2.9
	2-Et-4-MePh	1523.0	1520.0	3.0
	2-Et-5-MePh	1529.0	1534.4	−5.4
	2-Et-4,5-DiMePh	1656.0	1661.7	−5.7
	2-Et-5-*n*-PrPh	1706.0	1699.8	6.2
Group 3	2,6-DiMePh	1416.0	1415.8	0.2
	2-Et-6-MePh	1485.0	1485.8	−0.8
	2,6-DiEtPh	1549.0	1546.8	2.2
	2,3,6-TriMePh	1551.0	1546.4	4.6
	2,3,4,6-TetraMePh	1690.0	1683.4	6.6

or a decrease in *s* (eqns. 20, 21, 26 and 27). Comparative data on the influence of V_W and the connectivity index $^1\chi$ on the correlation coefficient of the corresponding equations are given in Table IX. The introduction of V_W into the molecular connectivity models instead of $^1\chi$ for all alkylphenol groups increases the correlation coefficient. In this instance, V_W and $^1\chi$ have opposite effects on the retention indices. There are analogous data in Table VIII (eqns. 29–32). The use of V_W instead of $^2\chi$ and $^3\chi$ also increases the correlation level of the corresponding equations.

A check of the practical applicability of the equations in Table VIII was made by successive exclusion of certain values of the retention indices from the total bulk of groups, by determining the coefficients of new equations using the models obtained and by the subsequent calculation of retention indices for these alkylphenols. The data obtained (Table X) indicate sufficient reliability of the proposed equations and their suitability for the prediction of retention indices and the identification of alkylphenols in different mixtures without the use of standards.

CONCLUSION

The combination of molecular connectivity indices of the first five orders with Van der Waals volumes permits structural models of alkylphenols to be obtained. These models describe adequately the peculiarities of their chromatographic behaviour.

The descriptor V_W contributes considerably to the molecular connectivity

models, and increases substantially the level of correlation of the corresponding equations. Among the connectivity indices, the influence of the order indices $^3\chi_p$ and $^3\chi_c$ and the total path and cluster index $^4\chi_{p+pc}$, which make important contributions to the retention indices, should be noted.

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Even–odd alternation effect in the regularities of chromatographic retention

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ABSTRACT

The influence of the structural factors due to difference in the configurations of molecules of homologues with even and odd carbon numbers on chromatographic retention is discussed. The relationship between retention and carbon number is expressed by a toothed (zig-zag) curve, *i.e.*, an alternation of retention values with transition from an even to an odd homologue is observed. Some data on the presence of the even–odd alternation effect in the regularities of changes in other physico-chemical properties of substances are given.

INTRODUCTION

When regularities in changes in the physico-chemical properties of substances belonging to homologous series are studied, account must be taken of the role of structural factors due to the peculiarities of the configurations of molecules with odd and even carbon numbers. This influence is manifested in a tooth-shaped (zig-zag) curve of the relationship between the physico-chemical characteristics and the carbon number of the molecule, z, *i.e.* there is an alternation of the values under consideration depending on z (whether it is odd or even). Recent studies have shown that a similar effect is manifested also in the regularities of changes in the characteristics of chromatographic retention within homologous series of solutes and stationary liquids. This paper reviews and discusses data available in the literature on this problem.

ALTERNATION OF PHYSICO-CHEMICAL CHARACTERISTICS OF HOMOLOGUES

The influence of carbon number was first revealed during investigations of the regularities of changes in melting temperatures, first for the homologous series of *n*-alkanes[1,2] and later for the homologous series of fatty acids[3]. In both instances the relationship between the melting temperature, T_{melt}, and carbon number z, is different for even and odd homologues. An equation was suggested[2] relating T_{melt} to z and taking into account the alternation effect:

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$$T_{melt} = 137.8 - \frac{2513}{5.141 + z - \varphi} \quad (1)$$

where $\varphi = 1$ for *n*-alkanes with odd z and $\varphi = 0$ for those with even z; 137.8 is the melting temperature of the limiting *n*-alkane, $(CH_2)_\infty$; 2513 and 5.141 are empirical constants characteristic of the homologous series in question.

Calculations of T_{melt} according to this equation are in good agreement with the experimental values. Similar equations might be suggested for other homologous series.

The alternation effect for liquid crystals is displayed not only with respect to the melting temperatures but also the temperatures of other phase transitions: smectic–nematic and nematic–isotropic liquid[4–8]. For example, it is observed in the dependence of the clarification temperature on the carbon number in alkoxy side-chains in a series of symmetrical azoxy ethers such as *p,p'*-(dialkoxy)azoxybenzenes used as stationary phases in chromatography.

The influence of z on the values of dipole moments, heats of crystallization, refractive indices and the size of fatty acid crystalline cells of methyl and dimethyl ethers of dicarbon acids was studied by Liepin' and co-workers[9,10] on the basis of literature data[3,11–14] and their own experimental data. A tendency for alternation of these physical constants, due to difference in the configurations of molecules with even and odd z, was obtained.

ALTERNATION OF GAS CHROMATOGRAPHIC RETENTION WITHIN A HOMOLOGOUS SERIES OF SOLUTES

The influence of z on the nature of intermolecular interactions in solutions is only slightly displayed, which is why for a long time it was not possible to evaluate this slight effect even with the help of such a highly efficient method as chromatography and, consequently, to reveal the corresponding retention anomalies. However, it was shown in Kováts' first paper[15] that the retention index was determined by logarithmic interpolation on the basis of logarithms of retention volumes of *n*-alkanes belonging to an even-carbon series, however, later this was considered unnecessary, and interpolation was carried out on the basis of the retention values of neighbouring *n*-alkanes.

With further developments in chromatography, it was ascertained that the dependence between the retention values and the carbon numbers of the molecules of solutes for a homologous series, which had previously been postulated to be linear, often deviates from linearity[16–20]. These deviations may be caused either by anomalies in the properties of solutes or by the sorption properties of stationary phases. A detailed study of this problem led to the conclusion that such deviations from linearity were mainly connected with the peculiarities of the molecular structure of the first members of the homologous series and were due to irregularities in the increment for a repeated fragment of the molecule. These deviations are most vividly manifested when polar solutes and/or polar stationary phases are used. The correlation between the chromatographic retention and the carbon number of the molecule, z, can be represented by the equation

$$\log t'_{R_z} = a + bz + cz^2 + \ldots \quad (2)$$

where t'_{R_z} is the adjusted retention time of a member of a homologous series with carbon number z and a, b and c are constants.

In our work[20], an effect was discovered, the essence of which is that the difference (ratio) of the retention values for a series of *n*-alkylbenzenes is minimal for the *n*-propylbenzene–ethylbenzene pair. Later this phenomenon was investigated by Soják *et al.*[21] and called "the propyl effect".

The simultaneous manifestation of the two "anomalous" effects, the propyl and the even–odd effects, is supported, in particular, by the retention data for alkylbenzenes on columns of squalane, citroflex (tributylacetyl citrate) and 1,2,3-tris(β-cyanoethoxy)propane[22–25].

The role of carbon number as a factor affecting the retention of homologues was first revealed when the *n*-alkanes–polyethylene glycol system was investigated[26]. An experiment on a highly efficient open-tubular column showed the relationship between the retention and z to be a broken line, *i.e.*, alternation of the chromatographic retention values takes place with the transition from an even to an odd member of a homologous series. Thus, strictly, this relationship should be depicted in the form of two curves, one of which corresponds to even and the other to odd values of z.

Eqn. 2 taking into account the alternation effect may be written as[27]

$$\log t'_{R_z} = a_t + b_t z + c_t z^2 + d_t z^3 + D_t \varphi_z \tag{3}$$

where a_t, b_t, c_t, d_t and D_t are constants and $\varphi_z = +1$ for even z and $\varphi_z = -1$ for odd z.

The alternation effect is slight, but it can be revealed when one considers the ratio of the adjusted values of the retention times, $r = t'_{R_z}/t'_{R_{z-1}}$, or the difference in the retention indices of neighbouring homologues, rather than the actual values of the retention time. When investigating the regularities of changes in retention with change in z for substances belonging to different homologous series, one should consider the ratio of the adjusted retention times or the difference in the retention indices of substances having the same carbon numbers, but belonging to two homologous series. The value of r is related to the difference in free enthalpies of evaporation from the stationary phase, $\delta(\Delta G^0)_z$, of two homologues with carbon numbers z and $z-1$ by the equation

$$\delta(\Delta G^0)_z = RT \ln (t'_{R_z}/t'_{R_{z-1}}) = RT \ln r \tag{4}$$

where R is the universal gas constant and T is the absolute column temperature. Hence the contribution of the carbon number can be represented in terms of energy units.

The distance between the lines on the plots for homologous series with even and odd z is characterized by the alternation criterion A_z[9,10,27]:

$$A_z = \log r_{z+1} - \log r_z = \log t'_{R_{z+1}} + \log t'_{R_{z-1}} - 2 \log t'_{R_z} \tag{5}$$

where r_z and r_{z+1} relate to the homologues with carbon numbers z $-$ 1, z and $z+1$, respectively. A_z depends on z and changes with the transition to each subsequent homologue.

Vigdergauz and Seomkin[27] succeeded in establishing the influence of z on the retention of *n*-alkanes, 1-bromoalkanes and 2-alkanones on a highly efficient column

with triethylene glycol dibutyrate, a stationary phase of moderate polarity. The investigation was carried out at 40 and 80°C. It was shown that the alternation effect increases in the series *n*-alkanes < 1-bromoalkanes < 2-alkanones and is clearly expressed by the relationship $\delta(\Delta G^0)_z = f(z)$. The toothed character of this relationship for 2-alkanones disappears with the transition to 1-bromoalkanes and then to *n*-alkanes. The relationship $\delta(\Delta G^0)_z = f(z)$ for *n*-alkanes is an almost straight line. The influence of the temperature is only manifested in the shift of the points on the plot, the shape of the curve remaining the same.

It is also of interest that $\delta(\Delta G^0)_z$ is smaller for 1-bromoalkanes with even than with odd carbon numbers, *i.e.*, there is a reverse regularity in the change in these values compared with *n*-alkanes. This effect can be accounted for by the role of the bromine atom in position 1, which makes the hydrocarbon chain longer, *i.e.*, the number of "chain-forming" atoms in 1-bromoalkanes with carbon number z should be considered to be equal to $z + 1$.

Fig. 1 illustrates the influence of carbon number on certain properties of *n*-alkanes[27]. The general shape of the dependence of the retention values on z is due to the regularities of changes in the saturated vapour pressures of the solutes (curve 1). The influence of z on the retention of *n*-alkanes (curves 2 and 3) is manifested more when polyethylene glycol 400 is used as the stationary phase than with triethylene glycol dibutyrate, which is a consequence of a more rigid molecular structure of the former. Curve 4 shows the dependence of the CH_2 group increment $\Delta l/l_2$ on z, where $\Delta l/l_2$ is the ratio of the difference in distances between the extreme carbon atoms for neighbouring homologues (Δl) to the length of the C–C bond (l_2). Curve 5 depicts the influence of z on melting temperature. The similar characteristics of curves 3 and 4 is

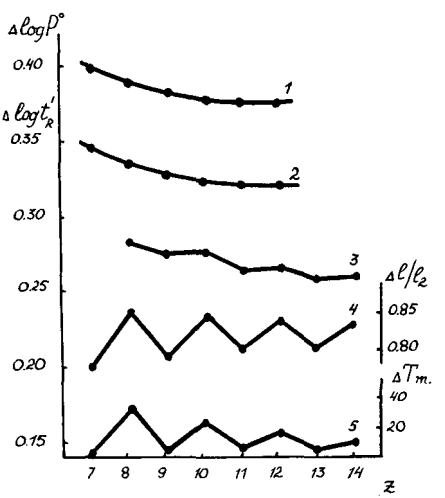

Fig. 1. Relationship between *n*-alkane properties and carbon number[27]. 1 = Homologous difference (difference for the neighbouring homologues) of logarithm of saturated vapour pressure, $\Delta \log P^0$, at 80°C; 2 and 3 = homologous difference of logarithms of adjusted retention times at 80°C on the columns (2) triethylene glycol dibutyrate and (3) polyethylene glycol 400; 4 = values of $\Delta l/l_2$, where Δl is the difference in distances between the extreme carbon atoms of the molecule for neighbouring homologues and l_2 is the length of the C–C bond; 5 = homologous difference of melting temperature, ΔT_{melt}.

evidence of a close relationship between the retention values and the molecular structure of the compounds in question.

The influence of z on the incremental value of a functional group in molecules representing different homologous series has been investigated[28]. *n*-Alkanes, aldehydes and ketones were chosen as solutes. The investigation was performed on an open-tubular column with triethylene glycol dibutyrate as the stationary phase. The capacity factor $k' = t'_R/t_0$, where t_0 is the hold-up time, served as a retention characteristic. The dependence of log k' on z has a form of a broken line and can be represented by an equation similar to eqn. 3.

The even–odd alternation factor was shown to increase in the series *n*-alkanes < bromoalkanes < aldehydes < ketones, the influence of this effect being the strongest with the first members of the homologous series and decreasing with increase in the length of the alkyl chain.

The calculation of retention increments on the basis of literature data[29] shows that alternation of ΔI values for the homologous series of methyl and chloromethyl ethers of *n*-C_5–C_{12} chlorocarbon acids on the open-tubular columns with the stationary phases SE-30 and Carbowax 20M has also been observed.

The alternation effect in the chromatographic retention of C_6–C_{11} *n*-alkanes is also manifested on packed columns with SKTFT-100 trifluoropropylmethylsilicone and pentaphenyl ether stationary phases[30]. The dependence $r = f(z)$ possesses a toothed character. Thus, for SKTFT-100 stationary phase the value of r is in the range 1.40–1.58 (the evaluation error is 0.01–0.04) and the values of $\Delta r = r_{z+1} - r_z$ is -0.11, 0.18, -0.14 and 0.14 for consecutive pairs of homologues.

The alternation effect becomes especially noticeable for polar solutes and polar stationary phases. In these instances alternation can be manifested even for packed columns of moderate efficiency. This phenomenon was studied, in particular, with fatty acids and their ethers[9,10,31]. The investigation was carried out at different temperatures on four stationary phases of different polarity, Apiezon L, Triton X-305, Carbowax 20M and diethylene glycol succinate (DEGS). A tendency for alternation of retention index ratios of the solutes in question was revealed on the columns with DEGS and Apiezon L. The alternation of these values was observed within the range $z = 3$–12, disappearing with further increase in the alkyl chain length.

The alternation effect of retention values of dimethyl esters of fatty dicarboxylic acids was also found on a column with the high-temperature liquid-crystal stationary phase (bis-*p*-ethoxybenzylideneamino)biphenyl[32]. The alternation is clearly expressed in the range C_4–C_8 whereas with increase in the number of CH_2 groups in the molecule it decreases.

The alternation criterion A_z has been calculated in order to resolve the question of the alternation of the retention values t'_{R_z}. A_z was calculated at 190°C on two types of stationary phase, polar (DEGS) and non-polar (Apiezon L). It was found that the alternation of t'_{R_z} is observed only on the polar phase within the range $z = 5$–12. A similar calculation also demonstrated the alternation effect of the retention values for dimethyl esters of dicarboxylic acids (190°C, DEGS stationary phase) within the range $z = 3$–6 and for free fatty acids (190°C, diethylene glycol adipate + H_3PO_4 stationary phase) within the range $z = 4$–10. As an example, Fig. 2 illustrates the alternation of the chromatographic characteristics of free fatty acids.

The study of the chromatographic behaviour of free fatty acids also showed that

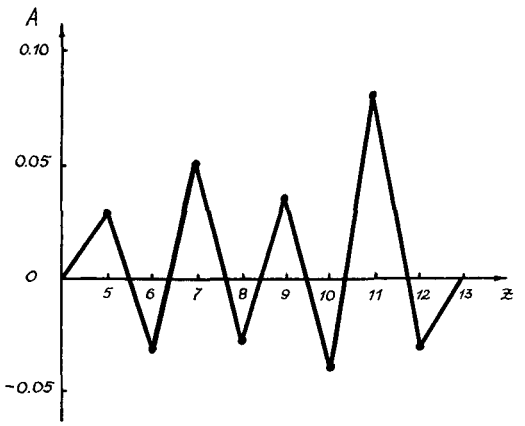

Fig. 2. Alternation of adjusted retention times of free fatty acids.

a decrease in the temperature of the column to 160°C results in the disappearance of the alternation effect; if the temperature is raised to 200°C, the effect is retained.

The alternation of the properties of a series of normal fatty acids is accounted for by the difference in molecular configurations of the even and odd homologues (Fig. 3). With monocarboxylic acids, if z is odd the methyl group is situated at one end of the molecule opposite the carboxyl group (B), and if z is even it is a methylene group (A). With dicarboxylic acids either the second carboxyl group or a methylene group is situated opposite the carboxyl group, depending on z. Therefore, if alternation is due to the configuration of the molecule, it is manifested more strongly with dicarboxylic acids and their derivatives. This is supported by experimental results[9,10]. Thus, if the alternation range of retention values is characterized by the difference $\Delta A_z = |A_{z+1}| + |A_z|$, it will be of the order 0.01–0.07 for free fatty acid methyl esters and 0.16 for the dimethyl esters of dicarboxylic acids. According to Liepin' and co-workers[9,10], a considerable role in the alternation phenomenon can also be played by the polymorphism of fatty acids, as there exist three forms, the molecules of which differ in the angle of location of the carbon atoms.

The alternation effect was also revealed when the thermodynamics of the dissolution of fatty alcohols in cholesteryl myristate was subjected to chromatographic study[33]. This effect might be due to the fact that the introduction of molecules of

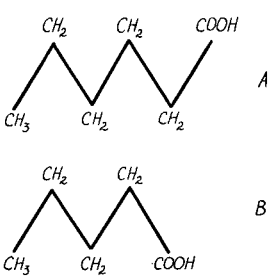

Fig. 3. Configuration of fatty acid molecules: (A) with even z; (B) with odd z[9].

non-mesogenic additives into the mesophase structure results in different efficiencies of alignment between the molecules and separate parts of the mesophase, this alignment being mainly a face-to-face interaction between the molecules of a liquid crystal and non-mesogenic additive. In this case an increase in the length of the alcohol carbon chain will lead to a decrease in the smectogenic nature of the molecules of cholesteryl myristate.

The selectivity of *p,p'*-(methoxyethoxy)azoxybenzene (MEAB) as a stationary phase with respect to C_{10}–C_{17} geometric and positional isomers of *n*-alkanes has been studied[7,34]. The dependence (Fig. 4) of the selectivity coefficient *r* on *z* is of a toothed character [*r* is the ratio of the adjusted retention times of *trans*-/*cis*-isomers at double bond position 2–8 (Fig. 4a, curves 2–8) and also the ratio of the adjusted retention times of the neighbouring *cis*-/*cis*- and *trans*-/*trans*-isomers (Fig. 4b)]. This confirmed the influence of *z* on the retention ratio of solutes having the same carbon number and belonging to different homologous series.

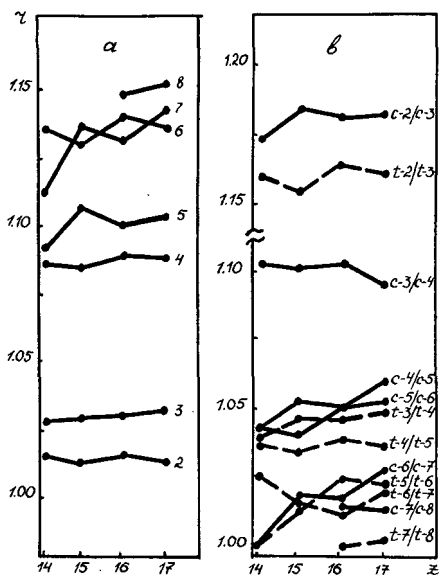

Fig. 4. Dependence of *r* on carbon numbers of C_{14}–C_{17} *n*-alkane molecules on a column with MEAB at 115°C[7]. (a) Separation of *trans*-/*cis*-isomers with the double bond in position 2–8; (b) separation of neighbouring *cis*-/*cis*- and *trans*-/*trans*-isomers.

ALTERNATION OF GAS CHROMATOGRAPHIC RETENTION VALUES ON COLUMNS WITH STATIONARY PHASES BELONGING TO THE SAME HOMOLOGOUS SERIES

The alternation effect is also manifested when the retention of the same solutes is studied on different stationary phases belonging to the same homologous series, in particular for the separation of close-boiling isomers on columns with liquid crystals of the nematic type, *e.g.*, azoxy ethers, as stationary phases. The influence of *z* can then be considered from different viewpoints: one can count (i) the total number of carbon

atoms in a liquid crystal molecule or (ii) the number of carbon atoms in both side-chains. In addition, one can examine the influence of z in one side-chain. Thus, when *m*- and *p*-xylenes were separated with symmetrical azoxy ethers as stationary phases[35–37], it was found that the dimethoxy derivative of azoxyanisole has a low selectivity, whereas the diethoxy derivative of azoxyphenetole has a much greater selectivity and consecutive homologues have nearly the same selectivity or a lower one (Fig. 5), However, if an asymmetric ether such as *p,p'*-(methoxyethoxy)azoxybenzene is used as a stationary phase the *meta–para* selectivity increases greatly and becomes higher than that with azoxyphenetole and its eutectic mixture with azoxyanisole[38].

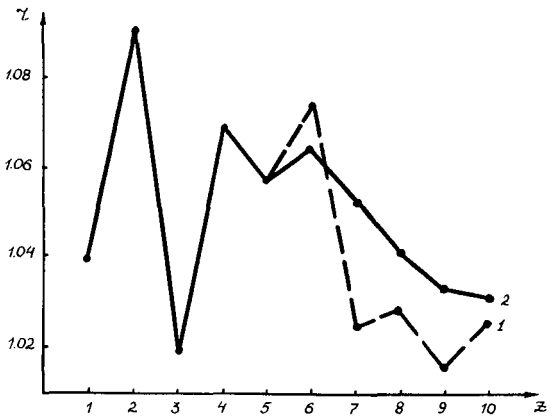

Fig. 5. Dependence of selectivity coefficient r on carbon number of the side alkoxy group of symmetric azoxy ethers for (1) smectic and (2) nematic mesophases.

The change in *meta–para* selectivity for a series of stationary phases prepared on the basis of symmetrical and asymmetric azoxy ethers has been investigated[7,39]. The selectivity coefficient r (in this instance the ratio of the adjusted retention times of *p*- and *m*-xylenes) was shown to experience alternation, which is due not only to the change in carbon number in the side-chains of azoxy compounds, but also to the dependence on the symmetry of the molecules. Note that, in general, asymmetric azoxy ethers possess a higher *meta–para* selectivity than the symmetrical compounds. MEAB has the highest *meta–para* selectivity in the series of asymmetric homologues.

An even–odd alternation effect in the retention of standard solutes (ethanol, methyl ethyl ketone, benzene and nitromethane) on liquid-crystal stationary phases belonging to the homologous series of nitroazoxy benzenes was revealed[40,41]. It should be mentioned that higher values of retention parameters are characteristic of odd- rather than even-carbon substituents.

THE EVEN–ODD ALTERNATION EFFECT IN LIQUID CHROMATOGRAPHY

The influence of z on sorption properties is also related to the corresponding regularities in the compositions of crude oils. Thus, the correlation of odd and even *n*-alkanes in present-day deposits and crude oils is shown to be different[42–45].

Molecules with an odd carbon number predominate in deposits, whereas in crude oils the prevalence of molecules with an odd z is slight, if any. To characterize this relationship, an "oddness index" was suggested[46], which is the ratio of the number of n-alkane molecules with an odd z ("odd" molecules, O) to that with an even z ("even" molecules, E). The O/E ratios in present-day deposits and crude oils are 2–5.5 and 0.9–1.2, respectively. The oddness index is used by many workers as an oil characteristic which makes it possible to evaluate the origins of crude oils. Another oil characteristic for identification purposes is the ratio of the contents of two alkanes with an isoprenoid structure in it, *i.e.*, pristane (2,6,10,14-tetramethylpentadecane) and phytane (2,6,10,14-tetramethylhexadecane)[47,48]. The prevalence of even and odd alkanes in crude oils (normal and branched) has long been related to the organic origin of petroleum in literature, whereas the fact that migration of crude oils through rocks, which are active solids, may lead to changes in oil composition was completely ignored[49]. To confirm this fact, one can use the results of studies[50,51] in which the alternation effect in the sorption properties of n-alkanes was investigated by liquid chromatography. It was shown that if a mixture of n-alkanes passes through a column of silica gel impregnated with hexane, the first fraction eluted is enriched with even-carbon hydrocarbons.

Fig. 6 depicts the change in the ratios of the concentration of the neighbouring n-alkanes with carbon numbers z and $z - 1$ when the initial (1) and final (2) fractions of the eluate were compared with the original mixture (0). The enrichment coefficient of the initial fraction of the eluate with the even-carbon alkanes was 1.24 for the chosen conditions.

It has been shown[51] that the filtration of crude oil through rock leads to the changes in the ratios not only of even/odd n-alkanes, but also of pristane/phytane, as compared with their ratios in the initial oil. A liquid chromatographic study was performed[52] close to conditions of fluid migration in nature, with activated silica gel impregnated with n-hexane as a porous medium, and the even–odd alternation effect

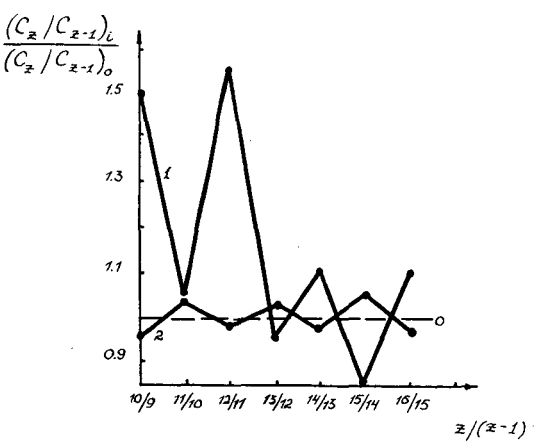

Fig. 6. Change in concentration ratios of the neighbouring n-alkanes with carbon numbers z and $z - 1$, when the initial (1) and final (2) eluate fractions are compared with the original mixture (0)[49].

was also revealed. The content of pristane in the initial fractions increased in comparison with that of phytane. In subsequent tests, this sorption effect gradually decreased and the filtrate composition became close to the original.

CONCLUSION

The even–odd alternation effect is manifested in the regularities of changes in the sorption properties of substances and, consequently, it must be taken into account when developing analytical methods and investigating related phenomena.

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Note

Quantitative structure-type analysis of hetero compounds–hydrocarbon mixtures

Analysis of S-heterocyclics and ketones from brown coal tars

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The working-up properties of brown coal tars and the quality of the products from it, such as paraffins, components for gasoline and diesel oils, lubricating oil components and electrode coke, are determined by the chemical composition of the tars. The hetero compounds present are converted during thermal pyrolysis or hydrogenation processes. The chemical conversions in the working-up processes are described only by the qualitative and quantitative structure-type analysis of the tar, charge substances and reaction products.

In previous papers[1,2] we reported on the development of an on-line method for the semi-preparative structure-type analysis of hetero compounds–hydrocarbon mixtures, such as tars and coal hydrogenation products. The application of this method to the determination of compounds that are typical of the nature of the coal conversion process[3,4] and to the determination of the chemical conversion of coals, tars and lubricating oil components in technical processes[3,5–7] was described.

S-Heterocyclics present in brown coal hydrogenation products are eluted by chromatography on normal phases according to their chemical properties with aromatics with the same number of π-electrons[3]. Thus, benzothiophene and its alkyl-substituted homologues are eluted together with dicyclic aromatics. The S-heterocyclics in this fraction were detected by capillary gas chromatography–mass spectrometry (GC–MS).

Gundermann *et al.*[8] and Nishioka *et al.*[9] separated S-heterocyclics from aromatic fractions obtained by the liquid chromatography of coal conversion products on an aluminium oxide–silica gel column forming complexes on a palladium chloride–silica gel phase ("fixed-phase extraction"). The palladium chloride–S-hetero cyclic complexes were eluted from this phase with dichloromethane and decomposed with ammonia. The S-heterocyclics were identified by GC–MS.

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The problem in this work was to separate the group of hetero compounds with similar elution properties to aromatic hydrocarbons by a fixed-phase extraction step in the on-line analysis method, and to identify the individual compounds by capillary GC.

EXPERIMENTAL

The separation scheme according to Zobel and co-workers[2,4] for on-line structure-type analysis was modified by inserting a complex formation step on a palladium chloride–silica gel phase column (100 mm × 8 mm I.D.) between the first precolumn and the silica-i-60-NH_2 phase column as a second precolumn. The modified separation scheme is shown in Fig. 1 and the resulting scheme for the semi-preparative high-performance liquid chromatographic (HPLC) apparatus in Fig. 2.

The palladium chloride–silica gel phase was prepared according to Nishioka *et al.*[9]. The eluents and the phase materials for the hydrocarbons and for the back-flushing of the hetero compounds are given in Fig. 1. The hetero compounds were separated into acidic (AHC), basic (BHC) and neutral hetero compounds (NHC) by application of an anion exchanger (Amberlite IRA-904) and a cation exchanger (Amberlyst A-15).

The palladium chloride–hetero compounds complexes were eluted from pre-column II with hexane–dichloromethane (90:10) and were decomposed on a small

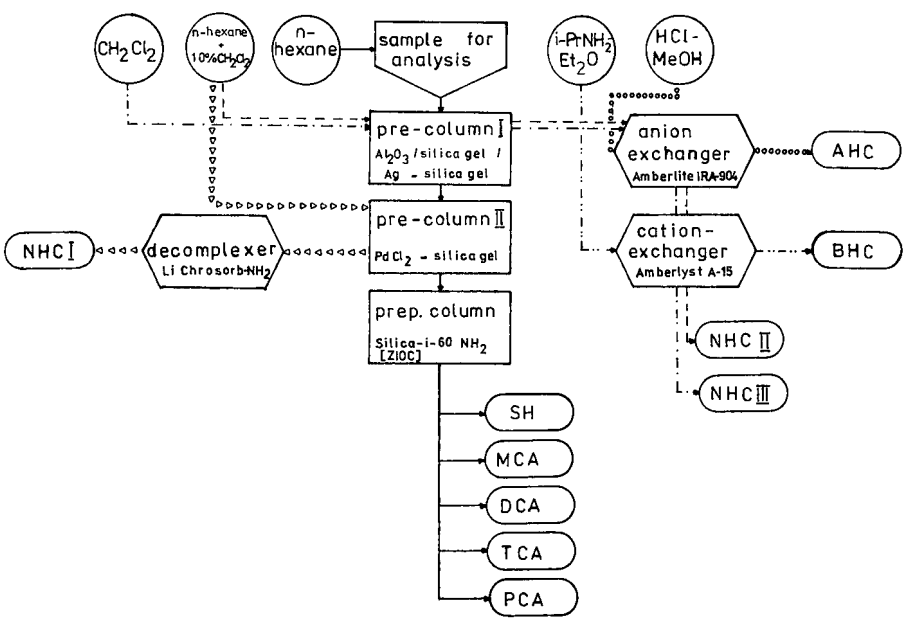

Fig. 1. Scheme of the structure-type separation by semi-preparative HPLC. SH = saturated hydrocarbons; MCA = monocyclic aromatics; DCA = dicyclic aromatics; TCA = tricylic aromatics; PCA = polycyclic aromatics; AHC = acidic hetero compounds; BHC = basic hetero compounds; NHC = neutral hetero compounds. Et = Ethyl; Me = methyl; i-Pr = isopropyl.

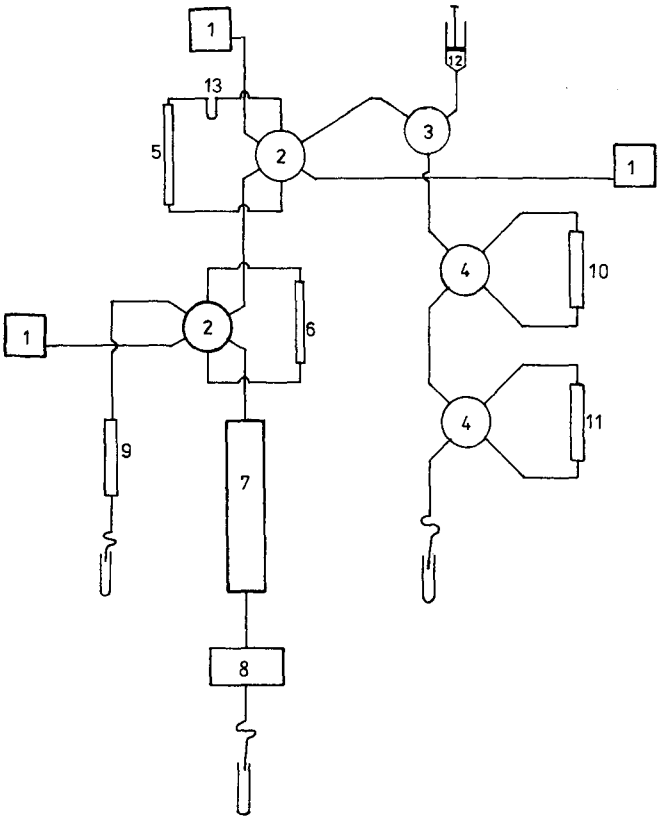

Fig. 2. Apparatus for on-line semi-preparative structure-type analysis. 1 = Pump; 2 = 6-port valve; 3 = 3-port valve; 4 = 4-port valve; 5 = precolumn I; 6 = precolumn II; 7 = separation column; 8 = differential refractometer; 9 = decomplexer column; 10 = anion-exchange column; 11 = cation-exchange column; 12 = injection syringe; 13 = sample loop.

decomplexing column filled with LiChroprep-NH_2 (100 mm × 8 mm I.D.). The eluate was evaporated in a stream of nitrogen and the amount of the hetero compounds was determined gravimetrically (NHC-I fraction).

The analytical rechromatography by HPLC, the quantitative interpretation of the structure-type analysis and the GC–MS conditions were described in a previous paper[4]. The individual compounds in the NHC-I fraction were identified by capillary GC with flame ionization detection (FID), flame photometric detection (FPD) and coupling with a mass spectrometer.

The following conditions for capillary GC were adopted: apparatus, Sichromat 1 with FPD and FID (Siemens); column, 15 m × 0.3 mm I.D. fused silica with DB-5; pressure, 0.18 MPa (nitrogen); injector temperature, 623 K; and temperature programme of the column oven, 313–573 K at 3 K/min.

TABLE I

STRUCTURE-TYPE COMPOSITION OF THE TAR OIL (b.p. >598 K) FROM THE DISTILLATION OF A BROWN COAL TAR

Structure type[a]	*Concentration (%)*	*Qualitative composition*
SH	34.2	Paraffins > olefins > naphthenes
MCA	9.5	Alkyl benzenes tetralenes, indanes
DCA	7.1	Naphthalenes, acenaphthenes > diphenyls, fluorenes
TCA	7.3	Phenanthrenes > anthracenes
PCA	2.7	Pyrenes, chrysenes
AHC	7.5	Phenols, indanols
BHC	1.5	
NHC I	5.8	Aliphatic ketones, benzothiophenes, dibenzothiophenes
NHC II	18.3	
NHC III	5.8	

[a] See Fig. 1.

RESULTS

The results obtained are demonstrated by the analysis of a tar oil (b.p. >598 K) from the distillation of a low-temperature brown coal tar. The structure-type composition and quality the principal groups obtained are given in Table I. The semi-preparative HPLC results are presented in Fig. 3.

By complex formation on the palladium chloride–silica gel phase the NHC-I fraction was separated, constituting 5.8% of the tar oil. Homologous series of peaks are shown in the capillary gas chromatogram (Fig. 4). These homologous series were identified by GC–MS as aliphatic ketones with carbonyl groups at the 2-, 3-, 4- and 5-positions in the alkyl chain. Phenanthraquinone is present in trace amounts. The alkanones are products of the thermal destruction of brown coal waxes[10,11].

Dibenzothiophene and its C_1–C_{3+}-alkyl-substituted homologues were detected and identified by GC–MS with method of the selective trace. With FPD a capillary gas chromatogram was obtained in which only the peaks of the S-heterocyclics were present (Fig. 5), from which their amount was determined.

The composition of the NHC-I fraction is given in Table II. The principal components are the alkanones, constituting 4.9% of the tar oil. Dibenzothiophene and its alkyl-substituted homologues constituted 0.85% of the tar oil. These represent 93% of the tar oil S-hetero compounds.

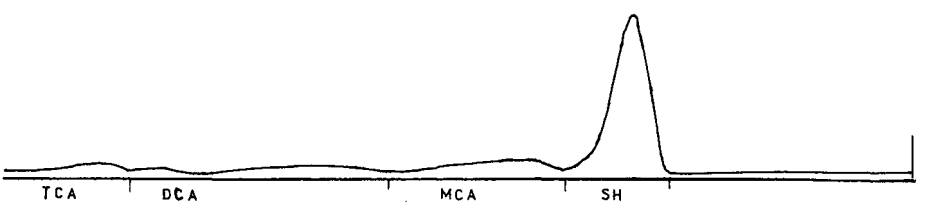

Fig. 3. Semi-preparative HPLC trace for a tar oil (b.p. >598 K) from the distillation of a brown coal tar.

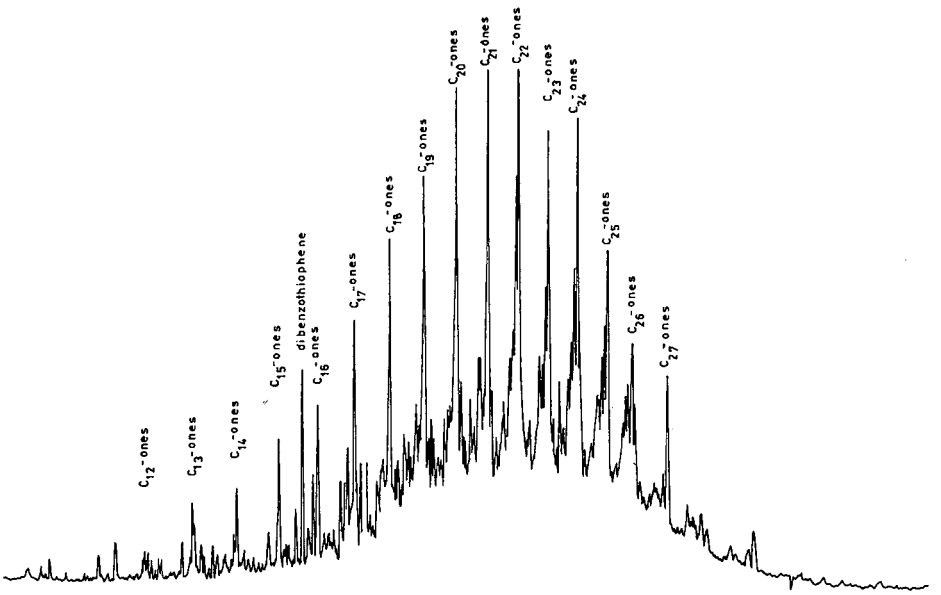

Fig. 4. Capillary gas chromatogram of the NHC I fraction with FID.

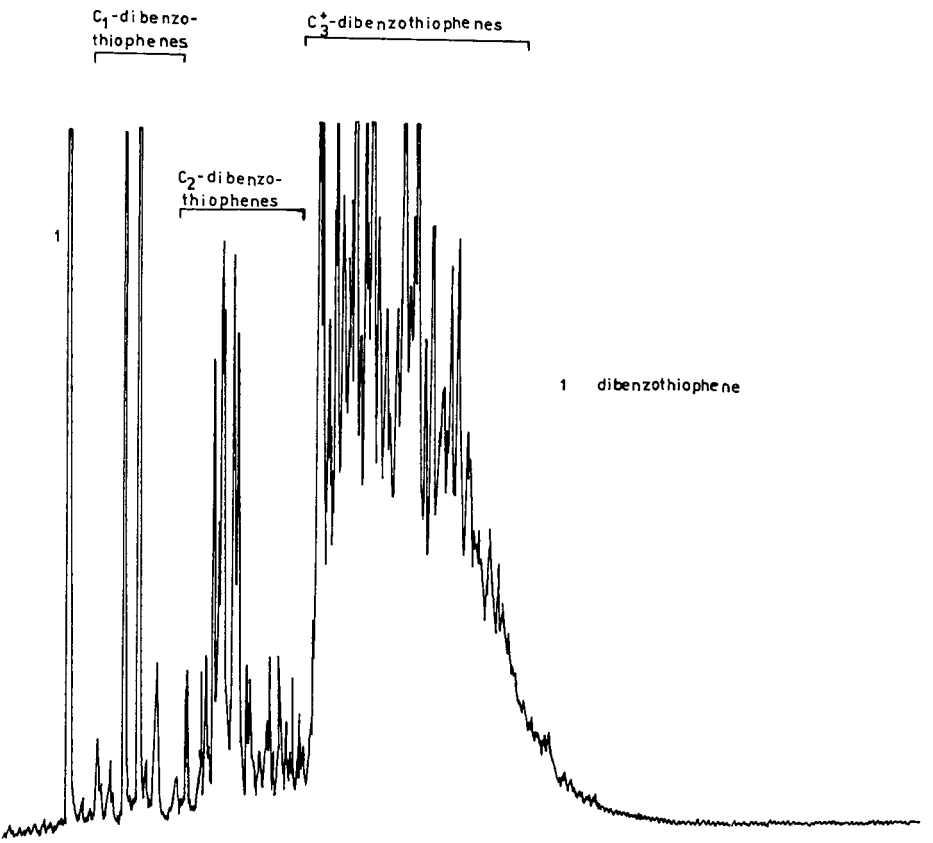

Fig. 5. Capillary gas chromatogram of the NHC I fraction with FPD.

TABLE II

COMPOSITION OF THE NHC I FRACTION BY CAPILLARY GC

Compounds	*Proportion of the fraction (%)*	*Proportion of the tar oil (%)*
Alkanones:	85.3	4.9
Alkan-2-ones	41.8	2.4
Alkan-3-ones, Alkan-4-ones, Alkan-5-ones	43.5	2.5
Dibenzothiophes:	14.7	0.85
Dibenzothiophene	0.8	0.05
C_1-Dibenzothiophenes	1.8	0.1
C_2-Dibenzothiophenes	2.6	0.15
C_3-Dibenzothiophenes	9.5	0.55

CONCLUSIONS

S-Heterocyclics and aliphatic ketones are separable as a group from brown coal tars by complex formation with palladium chloride. A requirement is that the hetero compounds with acidic and basic properties and with neutral OH groups and neutral N-bonding forms are separated previously.

The selectivity of palladium chloride complex formation, as described by Gundermann *et al.*[8] and Nishioka *et al.*[9], is not limited to the S-heterocyclics.

The group separation accomplished effects a clear improvement of the identification of S-heterocyclics and alkanones in tars.

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On-line chromatography with capillary columns and temperature programming

Determination of traces of 2-butenes in 1-butene and analysis of a reformate

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ABSTRACT

Two examples of automatic on-line chromatographic analysis with capillary columns and temperature programming are presented. The first is the detailed analysis of the effluent from a reforming unit. The sample is a C_4–C_{11} cut, mainly aromatic, which contains more than 200 compounds. It is shown that with automatic injection onto a capillary column, the resolution is as good as with manual syringe injection. Moreover, no discrimination between light and heavy compounds is observed. The second is the determination of ethylene, 1-butene (*ca.* 80%), traces of 2-butenes (less than 50 ppm), iso- and *n*-butane and C_6 olefins. This analysis was effected on a chromatograph equipped with two non-polar capillary columns and two detectors with different sensitivity ranges. The sample is injected simultaneously onto the two columns. On one detector the traces are analysed but the peak of the main component is saturated; on the other detector, the main component is analysed. The reproducibility of the retention times was checked, in both examples, in a 1-month test with automatic on-line injection.

INTRODUCTION

Automatic on-line process chromatographs are intended to operate automatically day and night without any intervention of the operator except for calibration of the apparatus. Up to now, almost all process chromatographs have worked under isothermal conditions and utilized packed columns, because the reproducibility of retention times is better under isothermal conditions than with temperature programming, and consequently the problem of the identification of peaks more easily solved, and injection onto packed columns is easier than onto capillary columns and the use of split injection is not necessary. However, the resolution of packed columns is worse than that of capillary columns. Therefore the analysis of complex mixtures with

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packed-column process chromatographs requires the use of column switching and often multi-oven chromatographs[1].

A few examples of the use of capillary columns have been described previously, mainly under isothermal conditions[2–4]. This paper describes the use of capillary columns and temperature programming for the automatic on-line analysis on two complex real examples: the complete analysis of gasoline and the determination of traces of 2-butenes in 1-butene.

Previously, a laboratory method was developed for the analysis of a reformate (gasoline from a reforming unit) and for the automatic calculation of the research and motor octane numbers (RON and MON, respectively) of this type of sample. The principle of the method is the separation of all the components of the sample on a capillary column with temperature programming, the automatic identification of those components, the calculation of their amount and the calculation of the octane numbers of the sample[5–7]. Such an analysis cannot be performed on packed columns. The transfer of this method to the automatic on-line analysis of reformate is described below.

Another application of on-line automatic capillary chromatography is the determination of traces components in gas samples in which the amount of the main component is greater than 70%. The principle of the analysis is the injection by gas valves of the sample simultaneously onto two capillary columns in the same gas chromatograph and connected to two different detectors. The sensitivity ranges of the two detectors are different: on the first the trace components are detected but the peak of the major compound is saturated, and on the second, the trace components are not detected but the peak of the main component is correct. This analysis is performed with temperature programming with a sub-ambient initial temperature.

Both on-line automatic methods were tested on pilot plants operating continuously.

EXPERIMENTAL

Analysis of a reformate

The samples were effluents from a pilot reforming plant operating continuously. The automatic on-line chromatographic analyses were carried out using a Hewlett-Packard 5890 gas chromatograph equipped with a Valco automatic liquid injection valve, a split injector and a flame ionization detector. The column was a 50 m × 0.22 mm I.D. wall-coated open tubular (WCOT) fused-silica capillary column coated with a non-polar methylsilicone stationary phase (PONA; Hewlett-Packard). The operating conditions were as described previously[5–7].

Integration of the peaks was carried out using a Hewlett-Packard 3392 integrator and data handling using a Hewlett-Packard HP 1000 A400 computer. Calibration, identification, quantification of components and determination of physical properties were performed using a Fortran program developed at the Institut Français du Pétrole.

Determination of traces of 2-butenes in 1-butene

The samples were effluents from a 1-butene pilot plant (Alphabutol IFP process) operating continuously. The automatic on-line chromatographic analyses were carried

out using a Delsi DI700 gas chromatograph equipped with two Valco automatic gas injection valves, two flame ionization detectors and a liquid nitrogen device for cooling the oven to sub-ambient temperature.

The columns were two 50 m × 0.2 mm I.D. WCOT fused-silica capillary columns coated with PONA. The carrier gas was helium and the operating conditions were as follows: −50°C for 2 min, increased from −50 to −15°C at 5°C/min and from −15 to 200°C at 10°C/min; the sensitivity ranges of the detectors were 10^{-11} A/mV (range 11, low-range detector) and 10^{-12} A/mV (range 12, high-range detector).

Integration of the peaks and data handling were carried out on a Hewlett-Packard HP 1000 A600 computer. The calculation of correct amount of each compound from the results given by the two detectors was performed using a Fortran program developed at the Institut Français du Pétrole.

RESULTS AND DISCUSSION

Analysis of a reformate: reproducibility

The automatic identification of the compounds in the reformate and the calculation of the RON and MON is based on the following principle. First, the *n*-alkanes are identified from their retention times; then, the retention indices with temperature programming for each peak are calculated and these calculated retention indices are compared with reference values in order to identify the components in the sample; and finally, using the amount of each compound and its RON and MON, the RON and MON for the sample are calculated.

The retention indices with temperature programming depend on the temperature of elution of the compound. This means that for automatic identification of all the compounds, they have to be eluted at almost the same temperature[5,6]. Consequently, the retention times of the *n*-alkanes should be adjusted so as to be in a previously defined range and they must remain constant from one analysis to another.

TABLE I

REPRODUCIBILITY OF RETENTION TIMES OF *n*-ALKANES DURING 5 WEEKS OF AUTOMATIC OPERATION OF THE REFORMATE OCTANE ANALYSER

n-Alkane	*Retention time (min)*				
	1st week	*2nd week*	*3rd week*	*4th week*	*5th week*
C_5	5.37	5.37	5.37	5.38	5.39
C_6	7.72	7.72	7.72	7.73	7.73
C_7	12.15	12.15	12.16	12.16	12.17
C_8	18.84	18.84	18.85	18.85	18.86
C_9	26.97	26.97	26.98	26.99	26.99
C_{10}	35.56	35.56	35.57	35.58	35.59
C_{11}	43.97	43.98	43.98	43.99	44.01
C_{12}	52.02	52.03	52.03	52.05	52.06
C_{13}	59.60	59.61	59.61	59.62	59.64
C_{14}	66.75	66.76	66.76	66.77	66.79
C_{15}	73.50	73.51	73.51	73.52	73.54

TABLE II

REPRODUCIBILITY OF THE AUTOMATIC ON-LINE ANALYSIS OF THE SAME REFORMATE SAMPLE DURING 5 DAYS AT A FREQUENCY OF 6 ANALYSES PER DAY

NA = *n*-Alkanes; IA = isoalkanes; N = naphthenes; A = aromatics; O = olefins.

Day	*Analysis*	*NA (%)*	*IA (%)*	*N (%)*	*A (%)*	*O (%)*	*Toluene (%)*	*Unknown (%)*	*RON*	*MON*
1	1st	8.98	22.37	1.89	65.34	1.36	14.11	0.08	94.3	84.9
	2nd	8.98	22.38	1.86	65.29	1.39	14.12	0.12	94.3	84.9
	3rd	8.96	22.36	1.90	65.34	1.36	14.14	0.08	94.3	84.9
	4th	8.94	22.29	1.86	65.45	1.35	14.14	0.13	94.3	84.9
	5th	8.90	22.18	1.87	65.57	1.37	14.17	0.12	94.3	84.9
	6th	8.90	22.23	1.93	65.53	1.39	14.16	0.04	94.4	84.9
2	7th	8.89	22.21	1.89	65.55	1.39	14.12	0.09	94.3	84.9
	8th	8.87	22.16	1.94	65.60	1.36	14.12	0.08	94.3	84.9
	9th	8.87	22.12	1.90	65.68	1.36	14.17	0.09	94.3	84.9
	10th	8.84	22.08	1.89	65.74	1.34	14.17	0.12	94.3	84.9
	11th	8.80	21.93	1.88	65.88	1.39	14.15	0.14	94.3	84.9
	12th	8.81	21.98	1.96	65.82	1.36	14.13	0.07	94.4	84.9
3	13th	8.80	21.98	1.92	65.82	1.36	14.12	0.13	94.3	84.9
	14th	8.80	21.97	1.93	65.83	1.36	14.15	0.13	94.3	84.9
	15th	8.83	22.02	2.00	65.76	1.36	14.16	0.04	94.4	84.9
	16th	8.80	21.93	1.98	65.93	1.35	14.17	0.04	94.4	84.9
	17th	8.79	21.94	1.99	65.92	1.35	14.19	0.03	94.4	85.0
	18th	8.79	21.88	1.94	65.98	1.33	14.19	0.08	94.4	84.9
4	19th	8.78	21.87	1.94	65.97	1.34	14.19	0.12	94.3	84.9
	20th	8.76	21.83	1.97	66.00	1.37	14.14	0.09	94.4	84.9
	21st	8.76	21.83	2.01	66.01	1.36	14.13	0.05	94.4	84.9
	22nd	8.76	21.85	2.02	65.99	1.36	14.15	0.04	94.4	84.9
	23rd	8.77	21.79	1.97	66.03	1.35	14.18	0.03	94.3	84.9
	24th	8.75	21.72	1.97	66.10	1.34	14.17	0.12	94.3	84.9
5	25th	8.75	21.71	1.98	66.14	1.34	14.20	0.09	94.4	84.9
	26th	8.73	21.67	2.05	66.13	1.36	14.18	0.09	94.4	84.9
	27th	8.70	21.68	2.05	66.20	1.35	14.17	0.04	94.5	84.8
	28th	8.74	21.68	2.07	66.09	1.39	14.17	0.04	94.4	84.9
	29th	8.72	21.61	2.02	66.19	1.39	14.18	0.09	94.4	84.8
	30th	8.71	21.59	2.01	66.24	1.37	14.17	0.09	94.4	84.9

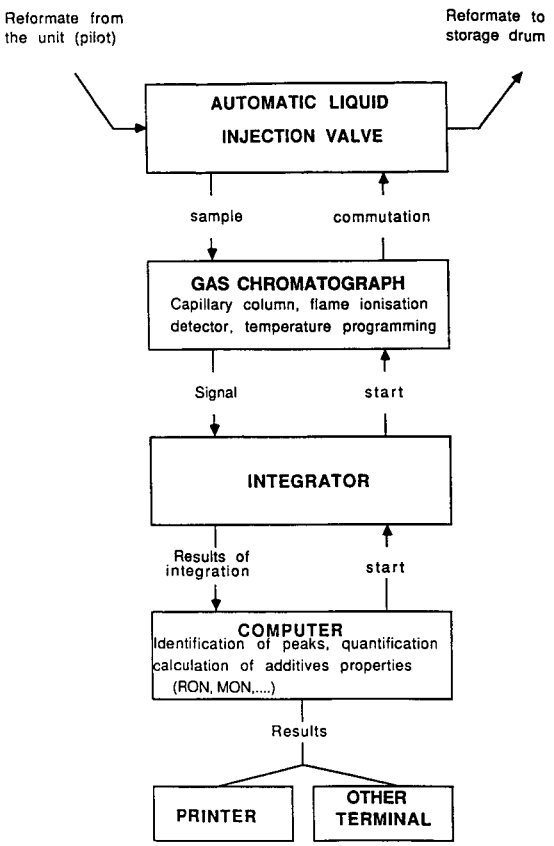

Fig. 1. Principle of the automatic on-line analysis of a reformate.

Table I gives the retention times of the *n*-alkanes in a calibration sample injected once a week when the apparatus is operating automatically with a frequency of eight analysis every 24 h. It can be seen that the main shift in retention time is less than 0.4%. This means that the *n*-alkanes in the sample are automatically identified from their retention times and the other compounds by their retention indices with temperature programming.

This was confirmed by the automatic on-line analysis during five days of the same sample circulating in a loop; the frequency of analysis was six every 24 h. The results are given in Table II. The small shift in the amounts of alkanes and aromatics is due to evaporation of a portion of the lighter compounds.

Fig. 1 shows the principle of the automatic analysis of a reformate.

Analysis of a reformate: on-line injection

A reformate is a complex mixture of hydrocarbons from C_4 to C_{11} (mainly aromatics), which contain more than 200 different components. The separation is performed on a non-polar capillary column; the operating conditions were previously optimized[5,6]. Owing to the complexity of the sample, it is very important to obtain the

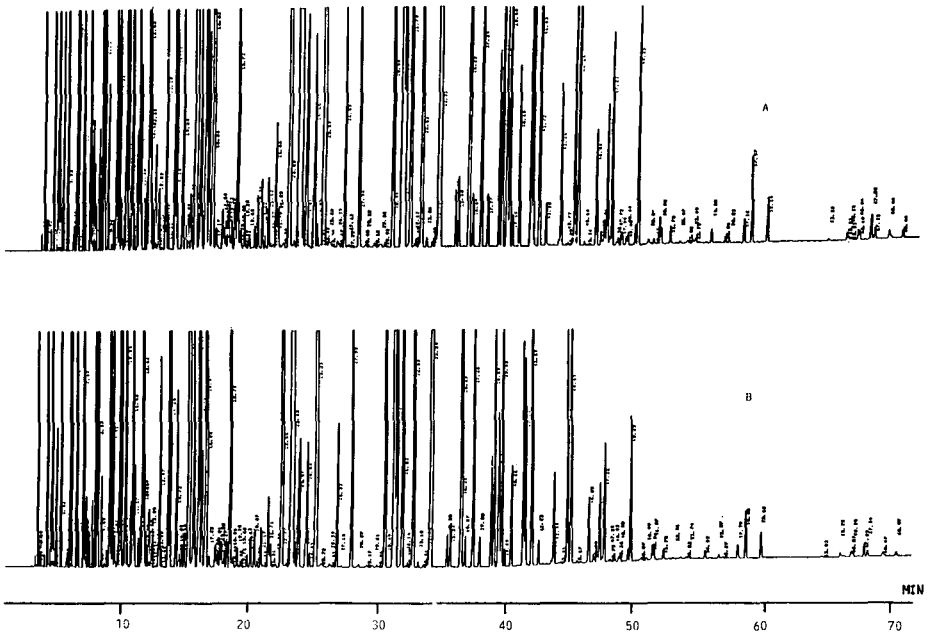

Fig. 2. Comparison of (A) syringe manual injection and (B) liquid valve injection capillary column chromatograms of a reformate.

same efficiency of the separation by valve injection and by syringe injection. The sample is injected in the liquid phase with a 0.5-μl injection valve in series with the split injector where it is vaporized. Fig. 2 shows that the separation is equally effective using automatic valve injection and manual injection.

The discrimination between light and heavy compounds during split injection is another known problem. With manual injection, no discrimination occurs over a large boiling point range, generally around 200°C. However, with automatic valve injection, the way in which the sample is vaporized is different, and the range without discrimination could be much smaller.

Consequently, it was checked that there was no discrimination due to the split injection. Table III shows the result of the analysis with automatic valve injection of a standard sample of *n*-alkanes in carbon disulphide. It can be seen that with valve injection there is no discrimination from C_5 to C_{13} but the amount of heavier compounds is subject to error. This means that the range without discrimination is shorter with automatic valve injection than syringe injection. However, the upper limit is higher than the final boiling point of the reformate. Consequently, the automatic analysis of a reformate can be performed by this method. This was confirmed by comparison of the analyses of the same sample using manual and automatic injection (Table IV).

Determination of a trace component in a major compound: principle of the method

On most commercially available gas chromatographs, the analysis of samples containing a major component and traces of other compounds is a problem because at

TABLE III

VALVE AUTOMATIC INJECTION OF A CALIBRATION SAMPLE CONSISTING OF THE SAME AMOUNTS OF *n*-ALKANES FROM C_5 TO C_{18} IN CARBON DISULPHIDE

Up to C_{13}, no discrimination occurs.

n-Alkane	*Concentration (%, w/w)*	*n-Alkane*	*Concentration (%, w/w)*
C_5	8.43	C_{12}	8.11
C_6	8.42	C_{13}	8.02
C_7	8.21	C_{14}	6.97
C_8	8.23	C_{15}	5.95
C_9	8.32	C_{16}	4.92
C_{10}	8.19	C_{17}	4.31
C_{11}	8.23	C_{18}	3.68

a high range of the detector the peak of the major component is saturated (Fig. 3) and at a lower range the trace components are not detected. Also, if the trace components are eluted close to the major component, it is not possible to program an automatic change of range.

This problem can be solved by analysing the sample simultaneously on two similar columns, connected to two different detectors, in the same chromatograph. One of the detectors is at a high range in order to detect the trace components and the other is at a low range in order to avoid the saturation of the peak of the major component. To calculate the exact amount of each component, the sample must also contain a compound present in a medium amount, the peak of which is not saturated in the high-range detector and which is detected by the low-range detector. This

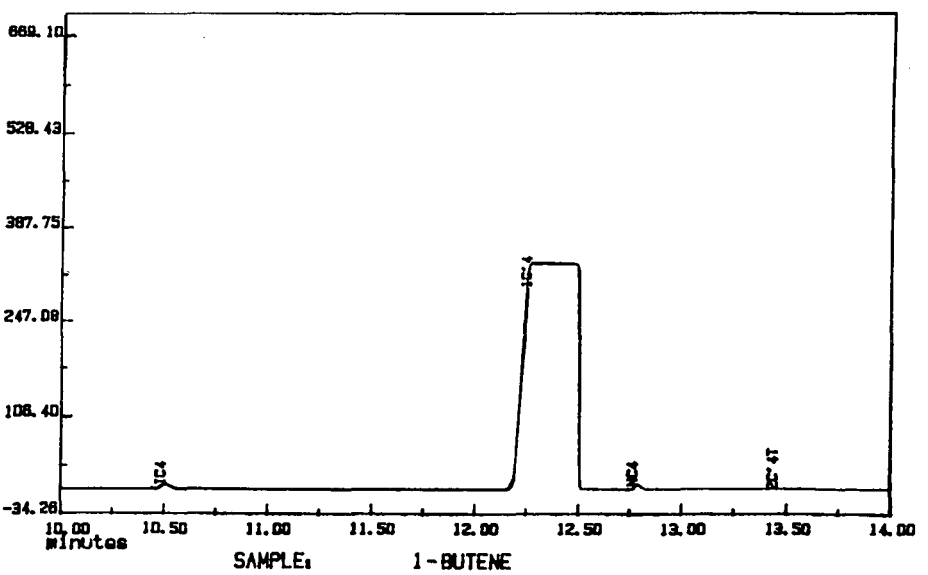

Fig. 3. Chromatogram of the saturated peak of 1-butene.

TABLE IV

COMPARISON OF ANALYSES OF THE SAME REFORMATE WITH ON-LINE AUTOMATIC INJECTION AND NORMAL SYRINGE INJECTION

Compounds	*Automatic valve injection*											*Manual syringe injection*										
	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}	*Total*	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}	*Total*
n-Alkanes	0.01	0.48	2.48	2.60	2.08	0.54	0.06	0.02	0.00	0.00	8.28	0.01	0.48	2.47	2.58	2.07	0.54	0.06	0.02	0.00	0.01	8.23
Isoalkanes	0.00	0.00	3.38	6.28	7.60	2.55	0.32	0.00	0.00	0.00	20.14	0.00	0.00	3.37	6.26	7.56	2.54	0.31	0.00	0.00	0.00	20.05
Naphthenes	0.00	0.00	0.00	0.29	0.30	0.13	0.00	0.00	0.00	0.00	0.73	0.00	0.00	0.00	0.28	0.30	0.15	0.00	0.00	0.00	0.00	0.74
Aromatics	0.00	0.00	0.00	4.17	19.69	27.48	16.09	2.47	0.16	0.09	70.15	0.00	0.00	0.00	4.14	19.59	27.46	16.20	2.51	0.17	0.15	70.22
Olefins	0.00	0.01	0.10	0.15	0.24	0.13	0.01	0.00	0.00	0.00	0.64	0.00	0.01	0.10	0.15	0.24	0.16	0.01	0.00	0.00	0.00	0.68
Unknown	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.02	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	002	0.04	0.09
RON						97.99											98.03					
MON						88.7											88.7					

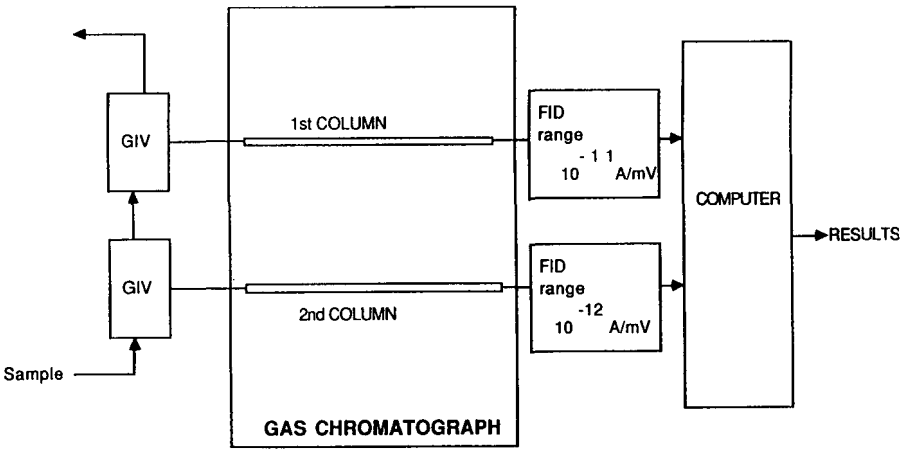

Fig. 4. Scheme of the procedure for the analysis of trace components in a major compound. GIV = Gas injection valve.

compound is used as an internal standard to correct the results from the high-range detector.

The factor K is calculated as K = %M on low-range detector/%m on low-range detector, where: M is the major component the peak of which is saturated on the high-range detector and m is the compound present in a medium amount which is detected on the low-range detector and the peak of which is not saturated on the high-range detector. Then, the correct amount of the major compound is calculated by the equation %M = $K \cdot$ %m on high-range detector. Finally, on the analytical report from the high-range detector, the incorrect value of the amount of M is replaced by the correct value and a normalization to 100 is performed.

One of the advantages of this method over, *e.g.*, injection of an external standard, is that it can be easily automated. The sample is injected simultaneously onto the two columns by automatic injection valves. The integration of the peaks from the two detectors is performed on the same mini- or personal computer, which can automatically calculate the correct amount of each compound immediately after the integrations. The scheme of the procedure is shown in Fig. 4.

Determination of traces of 2-butenes in 1-butene

An example of the application of the method is the automatic analysis of effluent from a unit that produces pure 1-butene from the dimerization of ethylene. At the outlet of the reactor, the sample to be analysed contains 1-butene, the major component (*ca.* 80%), remaining ethylene (a few percent) and by-products of the reaction, which are traces of 2-butenes (less than 50 ppm), *n*-butane (around 1000 ppm) and C_6 olefins. The main interest in this process for 1-butene production compared with other types of processes is the very low small amount of 2-butenes obtained as by products. Therefore, it is very important to determine the correct amount of 2-butenes and to monitor it continuously.

A correct separation of 80% of 1-butene and traces of 2-butenes and *n*-butane cannot be easily performed on packed columns. Consequently, this analysis is carried

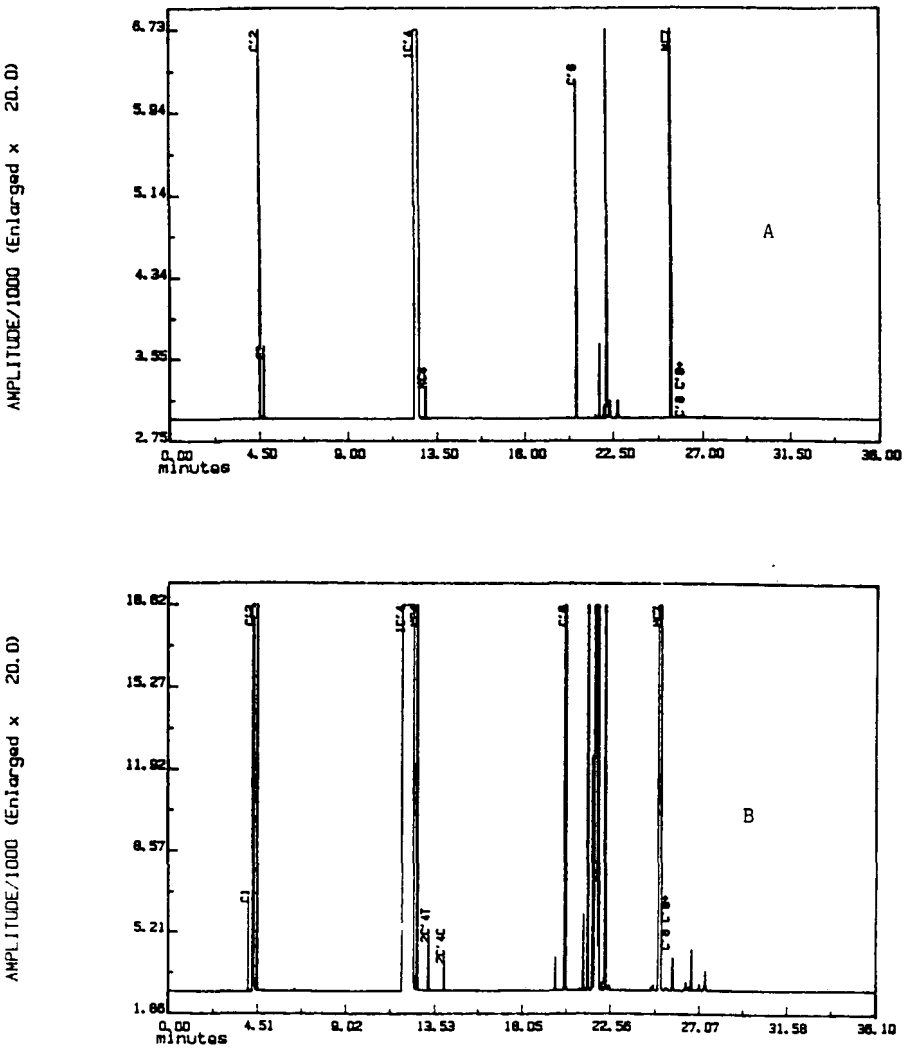

Fig. 5. Determination of traces of 2-butenes in 1-butene: (A) chromatograms of a 1-butene sample with 10^{-11} A/mV range and (B) 10^{-12} A/mV range flame ionization detector.

out on non-polar methylsilicone capillary columns (Fig. 5). The detectors which are used are flame ionization detectors, which are more sensitive than the thermal conductivity type.

The results of the analysis of a sample on a high-range detector, a low-range detector and the final result are shown in Table V. The effect of the saturation of the detector, which gives the amount of minor compounds too high, can be seen.

With a capillary column with a non-polar stationary phase, the separation of all C_4 hydrocarbons has to be done at sub-ambient temperature. Cooling the oven of the chromatograph is effected by liquid nitrogen. The use of a cryogenic cooling oven system could have produced poor reproducibility of the oven programming. During

TABLE V

ANALYSIS OF TRACES OF 2-BUTENES IN 1-BUTENE

Results with 10^{-11} and 10^{-12} A/mV range detectors and correct results after calculation.

Compound[a]	*% on 10^{-11} A/mV range detector*	*K = %M/%m on 10^{-11} A/mV range detector*	*% on 10^{-12} A/mV range detector*	*K · %M on on 10^{-12} A/mV range detector*	*Correct result (%)*
C_1	–		0.0251		0.0067
C'_2, M_1 saturated peak on 10^{-12} A/mV range detector	14.3173	13.5299	9.2730	53.7014	14.3617
C_2	0.1036		0.3933		0.1052
$1C'_4$, M_2 saturated peak on 10^{-12} A/mV range detector	79.0250	74.6787	66.9160	296.4072	79.2703
n-C_4	0.1057		0.4021		0.1075
$2C'_4$ *trans*	–		0.0277		0.0074
$2C'_4$ *cis*	–		0.0163		0.0044
3-Methyl-1-pentene internal standard (m)	1.0582		3.9691		1.0615
Other C'_6	1.8576		6.9950		1.8707
n-C_7	3.5170		11.9045		3.1837
Heavier compounds	0.0156		0.0779		0.028
Total	100		100	373.9196	100

[a] C_1 = methane; C'_2 = ethylene; C_2 = ethane; $1C'_4$ = 1-butene; *n*-C_4 = *n*-butane; $2C'_4$ *trans* = *trans*-2-butene; $2C'_4$ *cis* = *cis*-2-butene; C'_6 = hexene; *n*-C_7 = *n*-heptane.

5 weeks, the analysis of the sample was carried out automatically every 2 h; once a day, the retention times on the two columns of the main components were recorded (Table VI). The very low shift means that the reproducibility of the retention time is good enough for the automatic identification of the compounds by their retention times without modification of the calibration table. During this 5-week test, the automatic identification of the peaks by their retention times was performed and no error of identification was noticed.

CONCLUSION

It has been demonstrated that automatic on-line capillary column chromatography with temperature programming is possible even for very complex mixtures. For the two examples described, it would be very difficult and perhaps impossible to perform the same analysis with packed columns and/or under isothermal conditions; for a complete separation of a reformate there is no alternative to the use of capillary columns and temperature programming, and the separation of traces of 2-butenes in 1-butene is much easier on capillary columns. This means that the recent improvements in laboratory gas chromatography which involve the wide use of capillary columns and temperature programming will probably be widely used in the future for process chromatography.

TABLE VI

DETERMINATION OF TRACES OF 2-BUTENES IN 1-BUTENE

Reproducibility of retention times of main peaks with sub-ambient temperature programming during 5 weeks of automatic operation of the chromatograph (retention times in min).

Day	10^{-11} *A/mV detector*					10^{-12} *A/mV detector*				
	C'_2	$1C'_4$	n-C_4	C'_6	n-C_7	C'_2	$1C'_4$	n-C_4	C'_6	n-C_7
1	4.44	12.59	12.96	20.67	25.40	4.23	12.02	12.72	20.33	25.09
2	4.42	12.52	12.89	20.59	25.34	4.21	12.00	12.75	20.32	25.06
3	4.43	12.57	12.94	20.62	25.36	4.22	12.05	12.81	20.34	25.07
4	4.44	12.56	12.94	20.62	25.36	4.23	12.02	12.71	20.31	25.07
5	4.44	12.54	12.91	20.62	25.36	4.23	11.99	12.66	20.29	25.07
6	4.44	12.51	12.90	20.59	25.33	4.23	11.95	12.64	20.27	25.05
7	4.44	12.56	12.95	20.63	25.37	4.23	12.01	12.70	20.31	25.08
8	4.43	12.53	12.91	20.65	25.41	4.23	11.98	12.69	20.31	25.09
9	4.44	12.53	12.91	20.61	25.34	4.23	11.96	12.67	20.28	25.05
10	4.43	12.54	12.87	20.56	25.32	4.22	11.95	12.63	20.27	25.04
11	4.43	12.54	12.84	20.54	25.28	4.22	11.95	12.63	20.26	25.03
12	4.44	12.51	12.85	20.56	25.31	4.23	11.95	12.61	20.27	25.04
13	4.44	12.52	12.92	20.61	25.35	4.23	12.01	12.68	20.32	25.08
14	4.44	12.52	12.88	20.52	25.33	4.23	11.97	12.63	20.28	25.05
15	4.44	12.51	12.82	20.55	25.30	4.23	11.50	12.58	20.24	25.03
16	4.44	12.52	12.83	20.55	25.30	4.24	11.91	12.58	20.24	25.03
17	4.44	12.52	12.80	20.53	25.28	4.23	11.90	12.57	20.23	25.01
18	4.45	12.54	12.85	20.58	25.33	4.24	11.91	12.59	20.27	25.05
19	4.43	12.51	12.78	20.52	25.27	4.23	11.85	12.52	20.18	24.98
20	4.45	12.52	12.93	20.65	25.42	4.23	11.98	12.69	20.30	25.08
21	4.44	12.53	12.81	20.56	25.29	4.23	11.87	12.53	20.21	25.00
22	4.44	12.53	12.93	20.63	25.39	4.23	11.98	12.69	20.29	25.07
23	4.47	12.56	12.96	20.65	25.39	4.23	11.95	12.60	20.26	25.01
24	4.48	12.58	12.95	20.68	25.43	4.22	11.93	12.58	20.20	24.99
25	4.47	12.58	12.97	20.69	25.43	4.22	11.92	12.58	20.21	25.00
26	4.47	12.56	12.92	20.65	25.39	4.20	11.90	12.56	20.20	24.99
27	4.48	12.57	12.90	20.62	25.38	4.21	11.91	12.57	20.25	25.00
28	4.47	12.58	12.91	20.62	25.36	4.21	11.92	12.57	20.24	24.98
29	4.45	12.56	12.87	20.60	25.35	4.20	11.89	12.57	20.24	25.02
30	4.45	12.55	12.82	20.56	25.31	4.21	11.84	12.53	20.20	25.00
31	4.45	12.54	12.81	20.54	25.29	4.22	11.83	12.52	20.18	24.98
32	4.47	12.56	12.82	20.55	25.30	4.23	11.84	12.55	20.19	24.99
33	4.46	12.56	12.80	20.54	25.29	4.22	11.82	12.51	20.18	24.97
34	4.45	12.54	12.78	20.53	25.28	4.21	11.81	12.50	20.18	24.97
Higher gap[a]	0.05	0.08	0.19	0.17	0.16	0.04	0.24	0.31	0.16	0.12
Higher gap[b] (%)	1.1	0.65	1.5	0.8	0.6	0.95	2	2.4	0.8	0.5

[a] Difference between the higher and the lower value.
[b] Higher gap/medium value · 100.

It should be noted that the analyses described in this paper were carried out on laboratory equipment that is not explosion proof. However, equipment including a pressurized shelter to put inside the laboratory apparatus is now in progress and on-line tests in the refinery will take place in the near future.

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On-line chromatographic analyser for determining the composition and octane number of reforming process effluents

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ABSTRACT

An on-line analyser for reforming process effluents was developed from a commercial chromatograph and a computer. A temperature-programmed capillary column is used with software to make a detailed analysis (by automatic identification on the chromatogram) and to determine different physical properties (specific gravity, molecular weight, research and motor octane numbers, etc.).

INTRODUCTION

The growth in the demand for unleaded gasoline is a new challenge for refiners to produce high-octane gasoline during the next decade. In consequence, optimization of refining processes and development of new analysers are required. The on-line determination of octane number has become an important technique for refiners. The Cooperative Fuel Research engine is not well suited for on-line applications because it requires constant maintenance and frequents standardization, and consumes 0.5 l per test.

Numerous methods based on capillary gas chromatographic (GC) columns have been described during the last 20 years for determining different physical properties, especially the octane number, from detailed analysis[2–4]. Recently, several reports of octane-number determinations by spectroscopic techniques, such as nuclear magnetic resonance[5] and infrared spectroscopy[6] have appeared. Although these techniques have the advantage of being much faster than GC, they do not give a complete analyse of gasoline and their use in process control has not yet been developed or is difficult. GC is widely used in process control, but the development of an automatic analyser depends on the automatic identification of complex chromatograms (more than 200 peaks for gasoline chromatograms). With advances in the reliability of chromatographs and in GC data systems, this automatic identification has become feasible. A method using temperature-programmed retention indices has been described[7], with *n*-alkanes as reference peaks. These retention indices are reproducible by different chromatographs and commercial columns having the same characteristics

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if the retention times of *n*-alkanes are within ±5% of those used for setting up the reference table of retention times.

This paper describes an automatic on-line GC analyser for determining the composition and octane number of reforming process effluents.

EXPERIMENTAL

The instrumentation consists of a Hewlett-Packard (Palo Alto, CA, U.S.A.) apparatus package and software developed in our research institute.

Equipment and chromatographic conditions

The GC analyses were performed with a Hewlett-Packard HP 5890 gas chromatograph equipped with a flame ionization detector and with liquid valve injection by a split injector. Different effluents were selected by multi-port valves.

A fused-silica capillary column (50 m × 0.2 mm I.D.) cross-linked with a 0.52-μm film of OV-1 methylsilicone was used. The column oven temperature was increased from 40 to 200°C at 2°C/min with a final isothermal time of 20 min. The helium carrier gas flow-rate was set at 1.2 ml/min. The detector and injector temperature were held at 250°C. The splitting ratio was 1:170 and the sample size was 0.5 μl. The time between two analyses was 2 h.

Data integration was carried out using an HP 3392 A integrator and data handling using an HP 1000 computer (A 400 series).

Software package

The software was a Fortran program developed in our institute. It allows the analyser to run automatically while controlling different components, selecting effluents, calibrating, identifying and quantifying components and determining physical properties.

Control of the analyser. The analyser is computer controlled. At the outset the software checks to see if the chromatograph and integrator are ready. Then it selects an effluent with a purge period and, at the same time, it starts the injection, chromatograph and integrator. At the end of the analysis it transfers the data from the integrator to the computer.

Calibration. Calibration is carried out with a mixture of C_5–C_{18} *n*-alkanes. The software automatically identifies the reference peaks and creates a reference table of the retention times of *n*-alkanes, in which an identification zone is defined for each. This reference table is updated with the calibration mixture when the retention time of *n*-alkanes is between 60 and 100% of the identification zone.

Identification and calculation. n-Alkanes are identified from the reference table. Then, for each peak, an index I_{tp} is calculated, defined as

$$I_{tp} = \frac{t_{R,i} - t_{R,n}}{t_{R,n+1} - t_{R,n}}$$

where t is the retention time of compound i, eluted between *n*-alkanes with n and $n+1$ carbon atoms. The I_{tp} values calculated are compared with the reference index file. For each compound this file contains the index for the analytical conditions listed above

together with an identification window. The compounds are classified into five hydrocarbon groups: *n*-alkanes, isoalkanes, naphthenes, alkenes and aromatics.

Physical properties are determined by weight percentage analysis. For non-additive properties, coefficients are calculated by correlation with the properties measured for a batch of samples.

RESULTS AND DISCUSSION

Detailed analysis

More than 200 peaks were identified in gasoline up to C_{10} for saturates and C_{16} for aromatics (at a low level after C_{12}). Table I gives the final report on the aromatic group. The report also gives the distribution by carbon number and hydrocarbon group and the weight percentage of carbon-hydrogen. The results were compared with the PNA method [where P (paraffins) = alkanes, N = naphthenes and A = aromatics][8] for about 100 samples. The difference between the methods was less than 2% for the aromatic group.

Determination of physical properties

The specific gravity at 15°C and the molecular weight are calculated from the properties of pure components. For octane numbers the chromatogram is condensed into 45 groups according to the hydrocarbon group and carbon number of each compound. The coefficient for each group was adjusted to fit with the research octane number (RON) and motor octane number (MON) determined experimentally with the CFR engine; 150 samples of reformates selected for their different compositions by hydrocarbon groups were used for RON correlation. The octane number ranges of

TABLE I

FINAL REPORT ON THE AROMATIC GROUP

% (w/w)	*Component*	*% (w/w)*	*Component*
4.92	Benzene	0.12	1-Methyl-4-*n*-propylbenzene
22.56	Toluene	0.29	1,3-Dimethyl-5-ethylbenzene
5.47	Ethylbenzene	0.05	1-Methyl-2-*n*-propylbenzene
13.53	*m*-Xylene	0.17	1,4-Dimethyl-2-ethylbenzene
3.71	*p*-Xylene	0.16	1,3-Dimethyl-4-ethylbenzene
7.19	*o*-Xylene	0.29	1,2-Dimethyl-4-ethylbenzene +
0.26	Isopropylbenzene		1,3-Dimethyl-2-ethylbenzene
0.93	*n*-Propylbenzene	0.02	C_{10} aromatic
3.76	1-Methyl-3-ethylbenzene	0.06	1,2-Dimethyl-3-ethylbenzene
1.64	1-Methyl-4-ethylbenzene	0.22	1,2,4,5-Tetramethylbenzene
1.75	1,3,5-Trimethylbenzene	0.30	1,2,3,5-Tetramethylbenzene
1.41	1-Methyl-2-ethylbenzene	0.05	5-Methylindan
5.46	1,2,4-Trimethylbenzene	0.01	C_{11} aromatic
1.09	1,2,3-Trimethylbenzene +	0.01	Methylindene
	1-Methyl-4-isopropylbenzene	0.06	4-Methylindan
0.23	Indan	0.09	C_{11} aromatic +
0.02	Indene + 1 methyl-2-isopropylbenzene		1,2,3,4-tetramethylbenzene
0.09	1,3-Diethylbenzene	0.24	Naphthalene
0.17	1-Methyl-3-*n*-propylbenzene	0.01	C_{11} aromatic

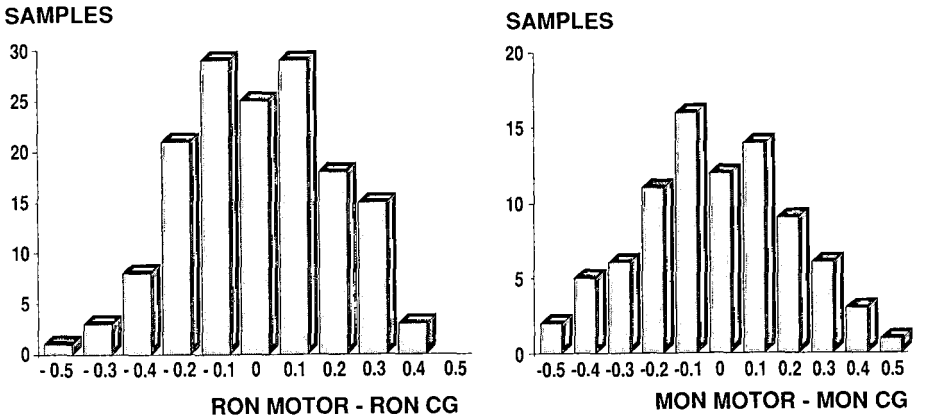

Fig. 1. Distribution of the samples versus the difference between the measured and determined research and motor octane numbers for determining the correlation.

these samples are 88–107 for RON and 78–95 for MON. Fig. 1 shows the distribution of the samples versus the difference between the measured and determined octane numbers.

Test of the analyser

The analyser was tested for 2 years on reforming and aromizing effluents with on-line and off-line configurations. The same capillary column was used, and calibration was done once per month. The difference between the measured and determined RON and MON was the same as for the correlation determination.

Intervention by the operator was required only for sample injection, *i.e.*, selection of different effluents (by modifying a sequence) or mixture calibration. Some chromatographic problems such as leaks or level of the baseline are given in the final report as a warning.

CONCLUSION

The development of an automatic capillary GC analyser has become feasible with advances in the reliability of chromatographs using a GC data system, and chromatographic data handling permits the direct determination of different physical properties. An automatic on-line capillary GC analyser was developed for determining the composition and octane number of reforming process effluents. With one analysis every 2 h, this analyser can control and optimize the operating conditions of a plant. However, the use of such an analyser on-line in a refinery requires the analyser to be explosion-proof. Such an operation is the next step of development. The best way for the analyser to be made explosion-proof is to locate the chromatograph, the integrator and the computer in a small pressurized shelter. The equipping of the shelter is now in progress, and an on-line test of the reforming unit of a refinery will take place in the near future.

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Note

Long-term use of liquid-crystalline stationary phase for separation and determination of polynuclear aromatic hydrocarbons in carbochemical products

Comparison of results obtained by different methods

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Modern technology for the isolation of pure anthracene and carbazole from anthracene oil[1] requires appropriate analytical techniques. Gas chromatography (GC) has been used for the determinaton of the components of such samples[2], particularly with the use of a liquid-crystalline phase[3,4]. The method[5] has been improved by modification of the support. In this work, the precision and accuracy of this method were evaluated by comparing the results with those obtained with polarography and UV spectrophotometry[6–8].

EXPERIMENTAL

Instrumentation

A MERA-ELWRO differential gas chromatograph, Type 504, with a flame ionization detector and Hitachi Model 356 and Pye Unicam PU 880 recording spectrophotometers were used.

Reagents

Chromosorb G AW (60–80 mesh) was obtained from Johns-Manville and N,N′-di(*p*-butoxybenzylidene)-α,α′-di-*p*-toluidine (BBBT) and silicone GE SE-30 from Applied Science Labs. Potassium carbonate was of analytical reagent grade.

The carrier and auxiliary gases were argon containing less than 0.01% oxygen impurity, air and hydrogen.

RESULTS AND DISCUSSION

The stationary phase consisted of a homogeneous mixture of liquid crystalline BBBT and a non-mesogenic phase, silicone elastomer GE SE-30. The support was modified by the addition of 2% of potasium carbonate.

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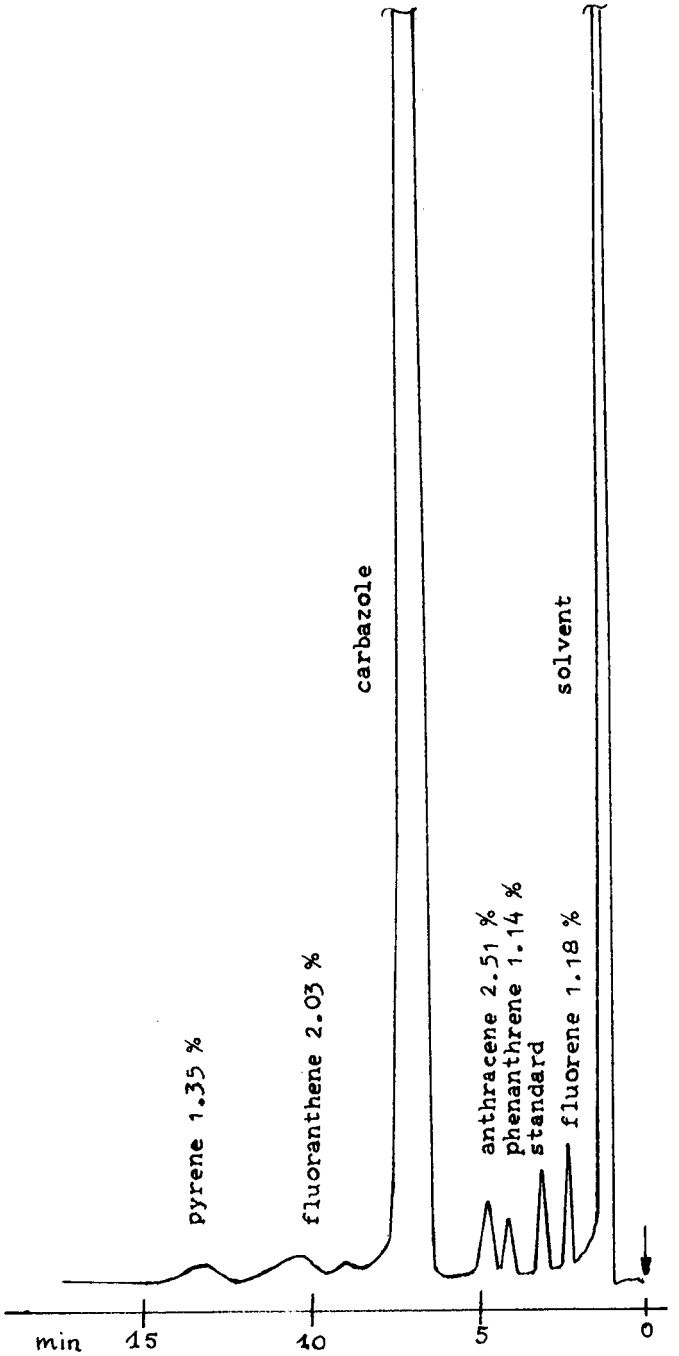

Fig. 1. Chromatogram of isolated carbazole. The separation was carried out on a glass column (190 × 4 mm I.D.) containing 3% BBBT + 2% GE SE-30 on Chromosorb G AW + 2% K_2CO_3 modifier. Column temperature, 230°C; carrier gas, argon at a flow-rate of 45 cm^3/min. Standard, triphenylmethane.

TABLE I

CHARACTERISTICS OF THE CHROMATOGRAPHIC COLUMN

Column parameters	*Characteristic*
Material	Glass
Stationary phase	BBBT + GE SE-30 (3% + 2%, w/w)
Support	Chromosorb G AW, 0.250–0.200 mm, + 2% K_2CO_3 modifier
Dimensions	1.9 m × 4 mm I.D.
Number of theoretical plates/m	1689
Lifetime	6 years (*ca.* 500 h/year of effective work)

The concentrations of particular compounds were calculated by using triphenylmethane as the internal standard. The amount of internal standard added was close to the percentage of the determined component. The calculations were made by using the following equation:

$$x_i = \bar{k}_{i/s} \frac{A_i / m_p}{A_s / m_s} \cdot 100$$

where x_i is the percentage of component i, $\bar{k}_{i/s}$ is the correction coefficient for component i, relative to the internal standard (s), A_i, A_s are the peak areas (mm^2) of component i and the standard (s), respectively, calculated as the products of the peak height and the width at half-height, and m_p, m_s are the masses (g) of the sample and the standard, respectively.

A good separation of fluorene, phenanthrene, anthracene, carbazole, fluoranthene and pyrene was obtained (Fig. 1). The carbazole peak, even at low concen-

TABLE II

RESULTS OF GC ANALYSES OF TECHNICAL SAMPLES

Sample	*Analyte*			
	Fluorene	*Phenanthrene*	*Anthracene*	*Carbazole*
Carbazole crude ($\bar{X}$ + S.D.)	11.5 ± 0.9	5.5 ± 0.25	10.2 ± 0.3	72.9 ± 2.0
Phenanthrene fraction ($\bar{X}$ ± S.D.)	17.4 ± 0.2	46.4 ± 0.3	6.2 ± 0.2	30.1 ± 0.3
Carbazole fraction	12.8	17.6	10.8	55.0
	17.4	28.1	9.9	40.6
	18.2	24.8	12.3	47.1
	16.7	33.7	7.9	25.8
Anthracene	5.8	11.2	75.9	6.9
	0.86	1.62	95.12 (as difference)	2.16

TABLE III

CHARACTERISTICS OF THE UV METHODS USED

Component	*Concentration range (%, w/w)*	*Analytical wavelength (nm)*
Anthracene	0.5–85	377: 374; 380
	85–99	377; differential method[7]
Carbazole	60–95	293; 290; 296; if phenanthrene content is below 3%
	0.5–5	293; 290; 296; sum of phenanthrene and carbazole
Phenanthrene	15–95	250; 247; 253; correction for anthracene (not more than 10%)
	0.5–5	293; 290; 296; sum of carbazole and phenanthrene

tration, is sharp. The column lifetime is more than 6 years (Table I). The time of analysis is short (*ca.* 15 min).

The precision was calculated on the basis of five independent determinations on the same sample. The results are given in Table II. Characteristics of the UV method are given in Table III.

The results of analyses of pure anthracene and standard mixtures obtained by GC and UV spectrophotometry are given in Table IV. The anthracene content obtained by the UV method is always lower, which may be indicative of the presence of impurities; the lower GC results for impurities may be due to adsorption on the column.

The GC results for the determination of anthracene and carbazole in various samples are given in Table V and compared with the results obtained by other methods. For the determination of anthracene in crude anthracene, GC, UV and polarographic techniques are suitable. The polarographic technique fails for aromatic

TABLE IV

COMPARISON OF RESULTS OF ANTHRACENE PURITY ANALYSIS IN STANDARD MIXTURES

Sample	*Component (%, w/w)*					
	Antracene		*Phenanthrene (GC)*	*Carbazole (GC)*	*Others (GC)*	*Phenanthrene + carbazole (UV)*
	GC[a]	*UV*[b]				
Anthracene, pure	99.7	98.1	traces	0.14	0.12	
Anthracene, pure + 2% carbazole	98.25	96.3	0.06	1.59	0.10	1.84
Anthracene, pure + 2% phenanthrene	98.06	96.3	1.72	0.05	0.17	1.80
Anthracene, pure + 1.5% carbazole + 1.5% phenathrene	96.8	95.2	1.33	1.79	0.09	3.11

[a] Anthracene determined as the difference (100 − sum of impurities) %.
[b] Anthracene determined as the main component by differential method.

TABLE V

COMPARISON OF RESULTS OF ANTHRACENE AND CARBAZOLE DETERMINATONS BY DIFFERENT METHODS

Sample No.	*Anthracene (%, w/w)*			*Carbazole (%, w/w)*	
	GC	*UV*	*Polarography*	*GC*	*Polarography*
1	42.8	42.6	42.3	13.5	15.7
2	44.0	44.5	43.7	13.1	15.2
3	42.1	41.2	42.3	13.9	14.5
4	41.4	42.5	43.3	14.7	16.6
5	11.2	11.3	—[a]	40.9	40.4
6	11.7	12.1	—[a]	43.8	44.0
7	12.6	11.8	—[a]	50.9	51.3
8	11.5	10.9	—[a]	45.1	47.5

[a] Results too high.

hydrocarbons in carbazole fractions. For carbazole determinations the GC and polarographic techniques are acceptable, although some systematic errors occur.

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Note

Gas chromatographic analysis of light hydrocarbons using aluminium oxide micro-packed columns

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Micro-packed columns filled with various adsorbents have been used successfully to separate different compounds. To increase the efficiency, packing materials with particle size up to 100 μm[1–6] (and even up to 175 μm[7]) have been used, namely silica gel[1–3], aluminium oxide[4], graphitized carbon black[5,6], molecular sieves[7] and Porapak[7]. Stainless-steel[4–7] and glass[3] columns with diameters not larger than 1 mm were used.

Previously we proposed the use of fused-silica capillary micro-packed columns containing activated coal[8], silica gel[9,10], molecular sieves[10] and Porapak[8], characterized by enhanced efficiency, convenience and possible utilization in solving a wide range of chromatographic problems. Fused-silica capillary micro-packed columns were used by Al-Thamir[11] (Chromosorb 102) to analyse natural gases and by Schindler and Wasserfallen[12] (Carbopak modified with Carbowax) to analyse polar compounds.

It was considered of interest to separate mixtures containing saturated and unsaturated C_1–C_6 hydrocarbons with fused-silica capillary columns packed with aluminium oxide.

EXPERIMENTAL

Fused-silica capillaries (length 40–200 cm, I.D. 0.32 and 0.55 mm) with polymeric coatings, both laboratory-made[13] and produced by Quartz et Silice (Nemours, France), and stainless-steel capillaries (50 cm × 1 mm I.D.) were used. The sorbents were coated on the columns by gas permeable partition using a binding material[14]. Aluminium oxide particles with diameters from 40–50 to 100–160 μm were used.

[a] Author deceased.

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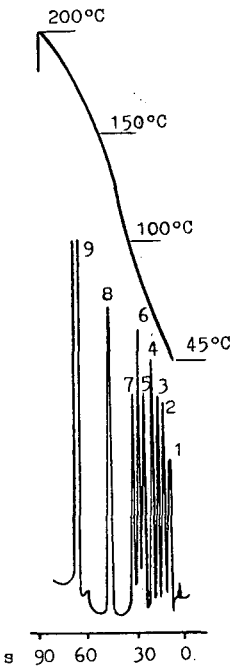

Fig. 1. Separation of C_1–C_6 hydrocarbons. Stainless-steel column, 50 cm × 1 mm I.D.; sorbent, aluminium oxide (63–100 μm); column temperature, programmed from 45 to 200°C; carrier gas, hydrogen; inlet pressure, 5 atm. Peaks: 1 = methane; 2 = ethane; 3 = ethylene; 4 = propane; 5 = propylene; 6 = isobutane; 7 = *n*-butane; 8 = *n*-pentane; 9 = *n*-hexane.

An LKhM-8MD gas chromatograph (Khromatograf, Moscow, U.S.S.R.) with a flame ionization detector was used. A stream splitter was used with the fused-silica columns. The carrier gas was nitrogen and the column temperature was 41–55°C.

Mixtures containing C_1–C_4 and C_1–C_6 were analysed.

TABLE I

CHARACTERISTICS OF CAPILLARY COLUMNS PACKED WITH ALUMINIUM OXIDE

Sorbate: isopentane.

Column material	*Column I.D., d_c (mm)*	*Al_2O_3 particle diameter, d_p (μm)*	*Minimum HETP, H_{min} (mm)*	*Coefficients in Van Deemter equation*	
				A (mm)	*C (s)*
Stainless steel	1	100–160	0.58	–	0.009
	1	63–100	0.27	0.25	0.0011
Fused silica	0.32	89–90	0.46	0.28	0.0009
	0.32	63–90	0.37	0.18	0.00045
	0.32	50–63	0.22	–	–
	0.32	40–50	0.19	–	–
	0.55	40–50	0.24	–	–

RESULTS AND DISCUSSION

A mixture containing nine C_1–C_6 hydrocarbons was completely separated with a stainless-steel column using temperature programming (Fig. 1). The analysis time was about 1 min; 40 s was sufficient to separate seven C_1–C_4 hydrocarbons.

Addition of isomeric butenes to the initial mixture led to incomplete peak separation at isothermal conditions. To increase the efficiency of separation it is expedient to use both smaller particles and smaller column diameters.

Data on the efficiency of the columns are given in Table I. The efficiency parameters of the fused-silica columns are higher than those of the stainless-steel column, owing to the smaller particle size and column diameter in the former. The lowest minimum height equivalent to a theoretical plate, H_{min}, is 0.19 mm for a column of I.D. (d_c) 0.32 mm packed with aluminium oxide particles of diameter (d_p) 40–50 μm. The increase in efficiency is first due to a decrease in the mass-transfer coefficient, C (coefficient in the Van Deemter equation). Higher carrier gas linear flow-rates (and, accordingly, a higher column inlet pressure) can be applied without any serious decrease in efficiency. Eddy diffusion, A, also decreases; note that A is equal to 2–3.5 particle diameters. The efficiency behaviour with both increase in carrier gas flow-rate and sample size was studied. Fig. 2 shows the dependence of $\sqrt{H}$ on sorbate mass, m, at the optimum carrier gas linear flow-rate, u_{opt}, and doubled carrier gas volume flow-rate, u. In both instances the dependence fits well the equation

$$\sqrt{H} = \sqrt{H_0} + \lambda m$$

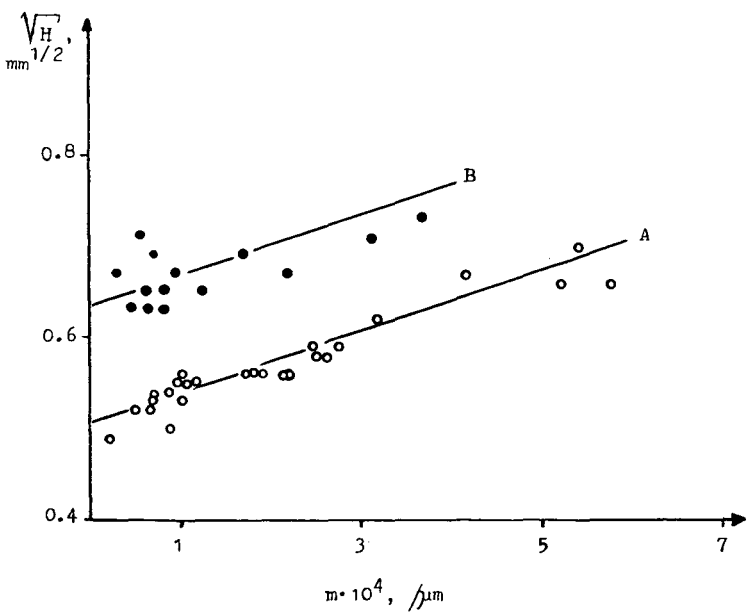

Fig. 2. Relationship between the sample (pentane) size, m, and efficiency characteristic, $\sqrt{H}$. Column, fused silica (73 cm × 0.32 mm I.D.); sorbent, aluminium oxide (63–90 μm); sample, n-pentane–n-hexane (1:9, w/w); column temperature, 77°C. (A) u_{opt} = 5.1 cm/s; (B) u = 11.8 cm/s.

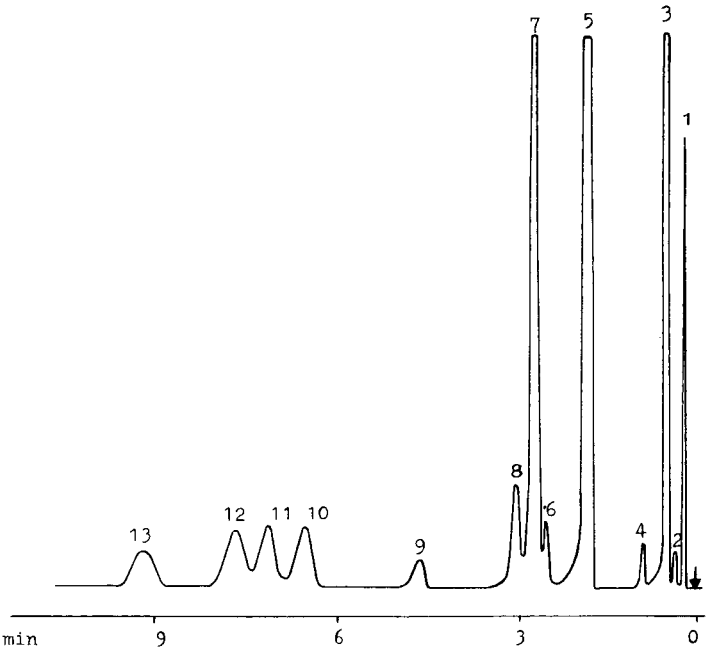

Fig. 3. Separation of saturated and unsaturated C_1–C_4 hydrocarbons. Column, fused silica (115 cm × 0.32 mm I.D.); sorbent, aluminium oxide (40–50 μm); column temperature, 45°C; inlet pressure, 1.0 MPa. Peaks: 1 = methane; 2 = ethane; 3 = ethylene; 4 = propane; 5 = propylene; 6 = isobutane; 7 = cyclobutane; 8 = *n*-butane; 9 = cyclobutene; 10 = 1-butene; 11 = *trans*-2-butene; 12 = isobutene; 13 = *cis*-2-butene.

where H_0 is the H value when $m = 0$ and λ is a proportionality factor[15]. H_0 increases with the increase in carrier gas flow-rate, but λ hardly changes.

To separate mixtures of saturated and unsaturated C_1–C_4 hydrocarbons containing cyclobutene and all the isomeric butenes, a fused-silica capillary column with small inner diameter packed with small particles was used (Fig. 3). The specific efficiency was not less than 5200 effective theoretical plates. The inlet pressure was about 1.0 MPa but when carbon dioxide was used instead of nitrogen a lower inlet pressure was sufficient.

In conclusion, a satisfactory separation of 10–13 components was achieved in about 7 min.

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CHROM. 22 161

Note

Determination of resin and fatty acids in pulp and paper mill effluents and white waters by gas–liquid chromatography

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The problems associated with effluent treatment to remove organic compounds are of paramount importance for the pulp and paper industry. Studies on the composition of organic components accumulating in white waters can contribute to their efficient removal and lower discharge loads.

Gas–liquid chromatography (GLC) has been successfully used for monitoring organic components. Usually the GLC analysis itself is preceded by the isolation of organic components from water samples[1–3]. Therefore, choosing correctly the isolation procedure and the separation of compounds into groups is a complex problem. When using multi-component effluents, the similar physico-chemical properties of the components to be determined can lead to the similarities in their chromatographic characteristics. On the other hand, when classical isolation procedures are used[4], different chemical conversions can occur, with further distortion of the results obtained.

In this work, the compositions of the organic components extracted with a mixed solvent (chloroform–diethyl ether) from white waters from thermomechanical pulping of spruce, the filtrate of a bleached kraft pulp suspension (pine) and effluents from a board mill and waste paper recycling plant (1:1) were investigated.

EXPERIMENTAL

The extracts of the water samples, prepared as described previously[5], were analysed a Tsvet series gas chromatograph equiped with a flame ionization detector using a 3 m × 3 mm I.D. stainless-steel column with a 5% DC 550 on Chromaton N AW HMDS. Helium was used as the carrier gas and the column temperature was 180°C.

RESULTS AND DISCUSSION

Fig. 1 shows that from white waters from thermomechanical pulping of spruce, C_{12}-C_{21} fatty acids (linoleic acid predominates), resin acids (abietic acid predominates) and aromatic compounds (of phenols vanillin predominates and of phenolic acids cinnamic acid predominates) are extracted. Fatty acids, aromatic acids, phenols

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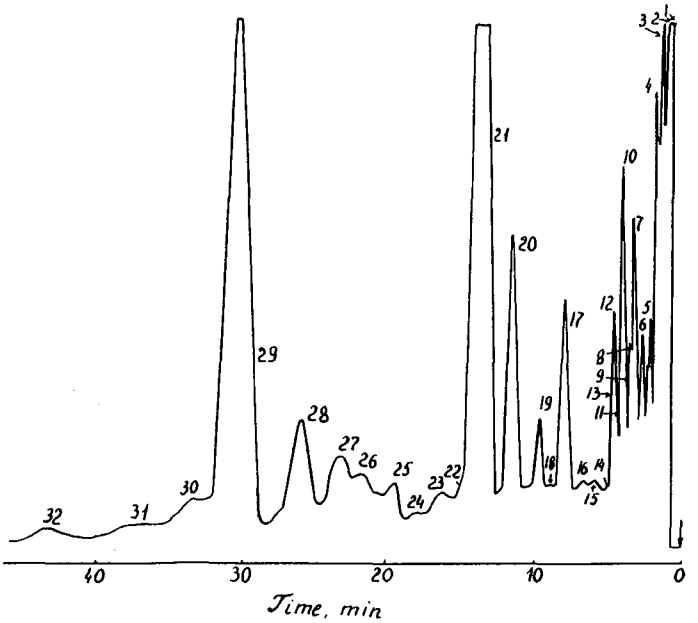

Fig. 1. Chromatogram of the extract of white waters from thermomechanical pulping. For conditions, see Experimental. 1 = *p*-Cresol; 2 = guaiacol; 3 = pyrocatechol; 4 = hydroquinone; 9 = isoeugenol; 10 = vanillin; 15 = pyrogallol. Acids: 5 = isolauric; 7 = lauric; 8 = cinnamic; 11 = vanillic; 12 = sebacic; 13 = cumaric; 14 = gallic; 16 = myristic; 18 = isopalmitic; 19 = palmitic; 20 = stearic; 21 = linoleic; 22 = arachic; 23 = isoheneicosanoic; 24 = pimaric; 25 = isopimaric + sandaracopimaric; 26 = levopimaric + palustric; 28 = dehydroabietic; 29 = abietic; 31 = neoabietic; 6,17,27,30,32 = unidentified.

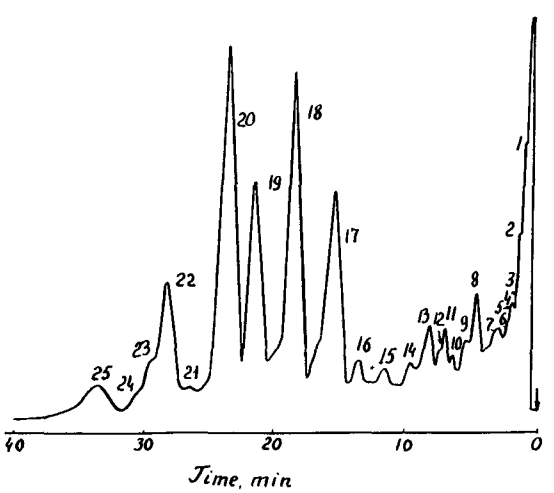

Fig. 2. Chromatogram of extract of board mill effluent. For conditions, see Experimental. 1 = *p*-Cresol; 2 = guaiacol; 4 = eugenol; 5 = isoeugenol. Acids: 3 = lauric; 6 = vanillic; 7 = isomyristic; 8 = myristic; 11 = palmitic; 12 = heptadecanoic; 13 = stearic; 14 = linoleic; 15 = arachic; 16 = pimaric; 17 = isopimaric + sandaracopimaric; 18 = palustric + levopimaric; 19 = dehydroabietic; 20 = abietic; 21 = neoabietic; 9,10,22–25 = unidentified.

and resin acids account for 29, 3, 20 and 30%, respectively, of the total amount of bound substances (fats, waxes or acid-soluble lignin). Farry acids, phenols, aromatic acids and resin acids account for 7, 15, 6 and 75%, respectively, of the total amount of free substances.

In the filtrate of a kraft pulp suspension (pine), C_{14}–C_{20} fatty acids (myristic acid predominates), aromatic compounds (pyrocatechol predominates) and resin acids (levopimaric and palustric acids predominate) were detected. Resin and fatty acids account for 41 and 39%, respectively, of the total amount of extracted substances.

TABLE I

GLC DETERMINATION OF ORGANIC COMPONENTS (%) IN WHITE WATERS OF THERMOMECHANICAL PULPING AND BOARD MILL EFFLUENTS

The measurement error of organic substances at a confidence limit of 0.95 is about 15%.

Type	*Component*	*Filtrate of kraft pulp suspension*	*Board mill effluent*	*White waters of thermomechanical pulping*
Phenols	*p*-Cresol	Traces	2.50	1.13
	Guaiacol	Traces	1.23	5.71
	Vanillin	1.20	n.d.[a]	8.35
	Isoeugenol	0.45	1.11	Traces
	Pyrogallol	n.d.	n.d.	0.24
	Eugenol	1.92	Traces	n.d.
	Pyrocatechol	3.60	n.d.	3.50
	Hydroquinone	n.d.	n.d.	5.90
Aromatic acids	Vanillic	2.80	Traces	Traces
	Cinnamic	n.d.	n.d.	1.18
	Cumaric	n.d.	n.d.	Traces
	Gallic	n.d.	n.d.	Traces
Aliphatic acids	Sebacic	8.05	n.d.	4.80
	Isolauric	n.d.	n.d.	2.82
	Lauric	n.d.	0.50	4.70
	Isomyristic	n.d.	0.99	n.d.
	Myristic	22.42	3.26	0.24
	Isopalmitic	n.d.	n.d.	Traces
	Palmitic	Traces	2.32	1.53
	Heptadecanoic	n.d.	Traces	n.d.
	Stearic	5.64	2.72	8.00
	Linoleic	Traces	0.79	27.10
	Arachic	2.80	1.09	Traces
	Isoheneicosanoic	n.d.	n.d.	0.41
Resin acids	Pimaric	0.87	1.53	0.12
	Isopimaric + sandaracopimaric	6.30	12.60	0.82
	Levopimaric + palustric	11.80	20.00	0.33
	Dehydroabietic	8.93	12.10	2.82
	Abietic	10.75	22.40	17.10
	Neoabietic	1.96	0.25	0.18
	Unidentified	10.51	14.61	3.02

[a] Not detected.

Board mill effluents (pine kraft pulp + secondary fibres) contained C_{12}–C_{20} fatty acids (lauric acid predominates), resin acids (abietic acid predominates) and aromatic compounds (*p*-cresol predominates). Resin acids and fatty acids account for 69 and 12%, respectively. The chromatogram of components isolated from board mill effluents shown in Fig. 2.

Tannins can be a source of aromatic compounds with free hydroxyl groups (pyrogallol and gallic acid series), and water-soluble lignin can be a source of substances with methoxy groups (vanillin and related compounds). The sources of fatty acids are fats, waxes, salts of acids and free acids.

Organic substances were determined by an internal standard procedure (with methyl stearate as the standard) and the results are summarized in Table I. Qualitative analysis was performed by comparing the elution times of substances on the chromatograms with those of standard substances.

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CHROM. 22 156

Analysis of some synthetic insect pheromones by gas–liquid chromatography

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ABSTRACT

The isomeric composition of insect pheromones belonging to primary olefinic alcohols and their derivatives was analysed by gas–liquid chromatography. The chromatographic behaviour of geometric isomers on liquid crystalline stationary phases was studied. The effectiveness and selectivity of the chromatographic columns and the Kováts retention indices of (*E,Z*)- and (*Z,Z*)-7,9-dodecadienyl acetates were determined and their dependence on temperature was investigated. Geometric and positional isomers of primary aliphatic alcohols and acetates with one and two double bonds can be separated on capillary columns with 1,2,3-tris-(β-cyanethoxy)propane, diethylene glycol succinate and cholesteryl *p*-methoxybenzoate.

INTRODUCTION

Many insect pheromones are primary olefinic alcohols, aldehydes or acetates containing 10–20 carbon atoms. Species specificity is conferred by variations of the structure, with differences in functional group, chain length, or changes in the position or geometry of the double bonds. Rigorously defined mixtures of isomers or geometrical purity of a synthetic product are often vital for maximum activity of pheromones. A technique capable of providing precise geometric definition is requied for the effective identification and utilization of pheromones.

The difficulty of separation of these isomers varies depending on the position and configuration of the olefinic bond and separations may become complex when diunsaturated compounds are involved. Capillary columns with high-polarity stationary phases have made possible the analysis of geometrical isomers of insect pheromones[1], but it is difficult to obtain good capillary columns with such stationary phases. Several workers have used liquid crystalline stationary phases, which exhibit unique selectivity towards geometric isomers[2–4].

This paper deals with liquid crystals as stationary phases for the efficient gas chromatographic analysis of unsaturated insect pheromones and the development of procedures for the separation of geometric and positional isomers by capillary gas chromatography.

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EXPERIMENTAL

A Tsvet Model 100 gas chomatograph equipped with a flame ionization detector and packed glass columns (3 m × 3 mm I.D.) was used. The liquid crystals (Reakhim) were deposited on the support from a chloroform solution, the solvent being evaporated in a rotary vacuum evaporater. The packing was then dried and screened. The packed columns were conditioned at 200°C and with a carrier gas (nitrogen) flow-rate of 20 ml/min for 7 h before use. The characteristics of the liquid crystals and columns prepared are given in Table I and II, respectively.

A Chrom Model 41 gas chromatograph equipped with a flame ionization detector, a stream splitter with a splitting ratio of 1:100 and a stainless-steel capillary column (50 m × 0.25 mm I.D.) coated with 1,2,3-tris(β-cyanethoxy)propane (TRIS) by the dynamic method was used[5]. The carrier gas was nitrogen at a flow-rate of 7.5 ml/min and the column temperature was 160°C.

A Biochrom Model I gas chromatograph equipped with a flame ionization detector, a stream splitter with a splitting ratio of 1:100 and glass capillary columns (50 m × 0.25 mm I.D.) was also used. The glass columns were coated with diethylene glycol succinate (DEGS) and cholesteryl *p*-methoxybenzoate (CMB) by the static method[6].

Efficiency, selectivity and retention data tests were carried out using a mixture of (*E,Z*)- and (*Z,Z*)-7,9-dodecadienyl acetates. Isomers were prepared in our institute.

TABLE I

CHARACTERISTICS OF STATIONARY PHASES

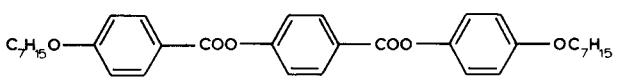
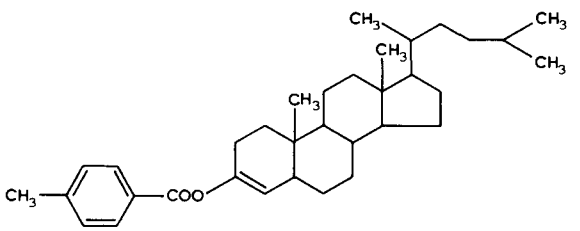
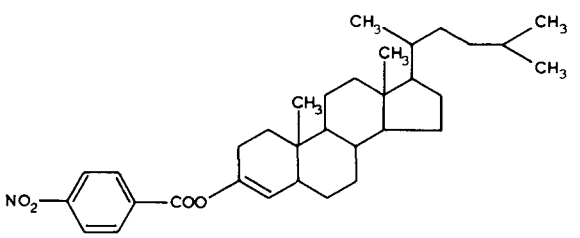
Phase	*Formula*	*Mesophase range (°C)*
I		120–195
II		178–230
III		185–235

TABLE II

CHARACTERISTICS OF CHROMATOGRAPHIC COLUMNS

Column	*Stationary phase*	*Support*	*Amount of stationary phase on support (%)*	*Column length (m) × I.D. (mm)*
1	I	Chromaton N-Super (0.125–0.16 mm)	7.5	3 × 3
2	II	Chromosorb W AW DMCS (0.15–0.18 mm)	10.0	3 × 2
3	III	Chromosorb W (0.18–0.25 mm)	10.0	3 × 3

The Kováts retention indices, I, column efficiency, N, selectivity, $r_{A/B}$, resolution, R_s, and capacity factors, k', of isomers were found from the following known relationships.

$$I_A = 100\ N + 100n \cdot \frac{\log\ t'_{R(A)} - \log\ t'_{R(N)}}{\log\ t'_{R(N+n)} - \log\ t'_{R(N)}}$$

where $t'_{R(A)}$, $t'_{R(N)}$ and $t'_{R(N+n)}$ are adjusted retention times of one of the isomers and alakanes with N and $N+n$ carbon atoms, respectively.

$$N = 5.54\left(\frac{t'_R}{w_{0.5}}\right)^2$$

where t'_R = adjusted retention time of (*E,Z*)-7,9-dodecadienyl acetate and $w_{0.5}$ is its peak width at half-height.

The selectivity of the stationary phases was expressed as the relative retention times of the isomers:

$$r_{A/B} = \frac{t'_{R(B)}}{t'_{R(A)}}$$

where $t'_{R(A)}$ and $t'_{R(B)}$ are adjusted retention times of (*E,Z*)- and (*Z,Z*)-7,9-dodecadienyl acetate, respectively.

$$R_s = \frac{t_{R(B)} - t_{R(A)}}{w_{0.5(A)} + w_{0.5(B)}}$$

where $w_{0.5(A)}$ and $w_{0.5(B)}$ are the peak width at half-height of the *E,Z*- and *Z,Z*- isomers, respectively.

$$k' = \frac{t'_R}{t_0}$$

where t_0 is the dead time. The dead time was determined according to the method of Peterson and Hirsch[7]:

$$t_0 = \frac{t_n t_{n+2} - t_{n+1}^2}{t_n + t_{n+2} - 2t_{n+1}}$$

where t_n, t_{n+1} and t_{n+2} are the retention times of members of the alkane homologous series.

RESULTS AND DISCUSSION

Stationary phases were tested in the range 100–200°C, so that the column was operated under supercooled mesophase conditions. At temperatures higher than 200°C liquid crystalline stationary phases showed bleeding.

The phase state of the liquid crystals changes with temperature. Table III shows measurements of the column efficiency, N, at column temperatures between 100 and 200°C and values of the difference between Kováts retention indices of (*Z,Z*)- and (*E,Z*)-7,9-dodecadienyl acetate. As the column temperature increases and the crystalline stationary phase tends towards the mesophase region, its solvation power increases and the column efficiency improves. After the column temperature rises above the mesophase range, the crystalline stationary phase melts and the column efficiency decreases.

The effect of column temperature and the state of the liquid crystalline stationary phase on the Kováts retention indices of geometric isomers is illustrated in Fig. 1. The Kováts retention indices measured on the cholesteryl stationary phases II and III increase linearly with increase in temperature. On phase I at temperatures

TABLE III

COLUMN EFFICIENCY (N) AND DIFFERENCE BETWEEN KOVATS RETENTION INDICES (ΔI) OF (*Z,Z*)- AND (*E,Z*)-7,9-DODECADIENYL ACETATE AT VARIOUS TEMPERATURES

Column temperature (°C)	*Column*					
	1		*2*		*3*	
	N	*ΔI*	*N*	*ΔI*	*N*	*ΔI*
100	986	6	–	−3	–	13
110	1758	19	23	−2	1391	14
120	4846	18	16	−2	1795	14
130	5016	18	27	−2	2004	14
140	4863	17	48	−1	2142	14
150	5160	16	94	−1	2210	15
160	4948	15	147	0	2211	16
170	4865	15	229	1	2237	16
180	1788	13	333	2	2075	16
190	2171	12	489	2	1996	17
200	2825	12	703	2	2039	17

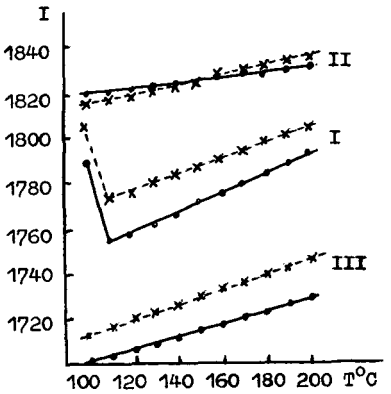


Fig. 1. Kováts retention indices of (○) (*E,Z*) and (×) (*Z,Z*)-7,9-dodecadienyl acetate on the liquid crystals I, II and III as stationary phases.

below melting point (120°C), the retention indices start to increase again. On phase II, (*Z,Z*)-7,9-dodecadienyl acetate eluates before the *E,Z*-isomer in the temperature range 100–150°C, but after it at temperatures above 160°C.

The difference between the retention indices of (*Z,Z*)- and (*E,Z*)-7,9-dodecadienyl acetate increases with increase in temperature on cholesteryl stationary phases II and III. The differences between those of geometric isomers increases with decreasing of temperature from 200 to 110°C and reaches 19 index units on the with nematic liquid crystal phase I, then it decreases sharply.

The selectivity of the stationary phases is demonstrated in Fig. 2. The values were directly dependent on temperature, and the highest selectivity towards isomers was exhibited by phase I.

The best resolving power was also exhibited by stationary phase I. The effect of its phase state on the resolution of (*E,Z*)- and (*Z,Z*)-7,9-dodecadienyl acetate is

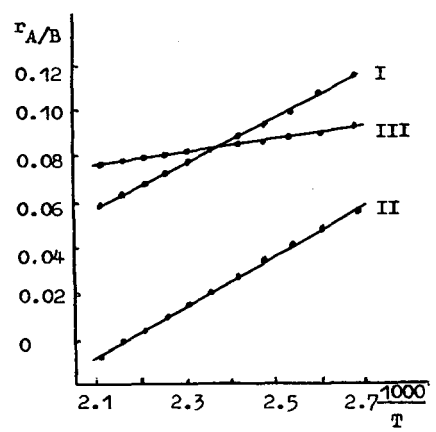


Fig. 2. Relative retentions of (*Z,Z*)- and (*E,Z*)-7,9-dodecadienyl acetate on the liquid crystals I, II and III as stationary phases.

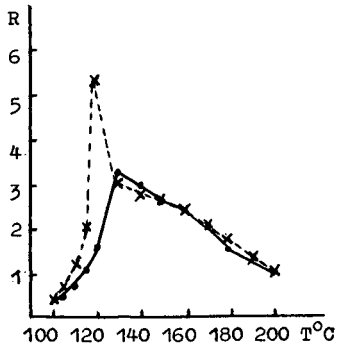


Fig. 3. Dependence of resolution, R_s, for (*E,Z*)- and (*Z,Z*)-7,9-dodecadienyl acetate on the column temperature with column (○) heating or (×) cooling. Colum 1.

illustrated in Fig. 3, which is a plot of the resolution R_s of the isomers against column temperature. Maximum resolution (R_s = 5) was achieved at the melting point (120°C) of 1,4-phenylene bis (4′-*p*-heptyloxybenzoate) when the column had been cooled.

Attempts to separate the isomers of monoolefinic insect pheromones on these packed columns failed. Fig. 4 illustrates the separation of the geometric isomers of European vine moth (A) and codling moth (B) pheromones on column 1 (3 m × 3 mm I.D.) with 10% nematic liquid crystal 1,4-phenylene bis(4-*p*-heptyloxybenzoate) on Chromaton N-Super, 0.125–0.16 mm. The carrier gas nitrogen flow-rate was 20 ml/min and the column temperature was 160°C.

Capillary columns were made in our laboratory. The efficiency of a column in separating isomers was expressed as the capacity factor, k', of isomers, the selectivity, r,

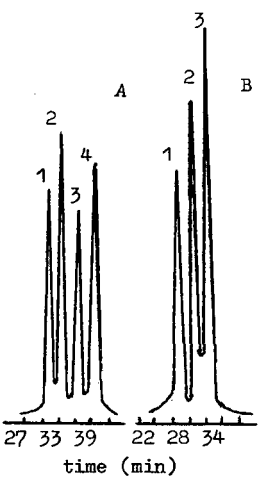


Fig. 4. Separation of geometric isomers of (A) European grape vine moth and (B) codling moth pheromones on column 1 (3 m × 3 mm I.D.) packed with Chromaton N-Super (0.125–0.16 mm) coated with 10% 1,4-phenylene bis(4′-*p*-heptyloxybenzoate). Nitrogen flow-rate, 20 ml/min; column temperature, 160°C. (A) 1 = (*Z,E*)-; 2 = (*E,Z*)-; 3 = (*Z,Z*)-; 4 = (*E,E*)-7,9-dodecadienyl acetates. (B) 1 = (*E,Z*)-; 2 = (*Z,Z*)-; 3 = (*E,E*)-8,10-dodecadienol.

TABLE IV

CAPACITY FACTORS (k') OF OLEFINIC ALCOHOLS AND ACETATES

Peak in Fig. 5	*Compound*	k'
1	(*E*)-7-Decenyl acetate	0.65
2	(*Z*)-7-Decenyl acetate	0.72
3	(*E*)-8-Decenyl acetate	0.73
4	(*Z*)-8-Decenyl acetate	0.83
5	(*E*)-9-Dodecenyl acetate	1.24
6	(*Z*)-8-Dodecenyl acetate	1.34
7	(*E*)-10-Dodecenyl acetate	1.35
8	(*Z*)-9-Dodecenyl acetate	1.37
9	(*Z*)-10-Dodecenyl acetate	1.52
10	(*E,Z*)-7,9-Dodecadienyl acetate	2.85
11	(*E,E*)-7,9-Dodecadienyl acetate	2.97
12	(*E,E*)-8,10-Dodecadienyl acetate	3.11
13	(*E,Z*)-7,9-Dodecadienol	3.63
14	(*E,E*)-7,9-Dodecadienol	3.81
15	(*E,E*)-8,10-Dodecadienol	4.04
16	(*Z*)-11-Tetradecenol	3.59
17	(*E*)-6-Hexadecenol	5.54
18	(*E*)-9-Hexadecenol	5.61
19	(*Z*)-6-Hexadecenol	5.87
20	(*Z*)-9-Hexadecenol	6.02
21	(*Z*)-11-Hexadecenol	6.09

and the resolution, R_s. The results obtained on a metal capillary column (50 m × 0.25 mm I.D.) with 1,2,3-tris(β-cyanethoxy)propane as stationary phase (N = 38 000 theoretical plates) are presented in Tables IV and V. The column is effective for the separation of geometric and positional isomers of olefinic pheromones. The value of

TABLE V

SELECTIVITY ($r_{A/B}$) AND RESOLUTION (R_s) OF GEOMETRIC AND POSITIONAL ISOMERS OF OLEFINIC ALCOHOLS AND ACETATES

Separated compounds (peaks Fig. 5)	$r_{A/B}$	R_s	*Separated compounds (peaks Fig. 5)*	$r_{A/B}$	R_s
1–2	1.14	2.74	6–7	1.00	0.00
3–4	1.16	3.25	8–7	1.01	0.00
1–3	1.13	2.24	5–9	1.23	4.58
2–4	1.15	2.80	10–11	1.05	1.10
2–3	1.01	0.00	11–12	1.04	1.26
5–8	1.10	2.00	13–14	1.05	1.15
7–9	1.13	2.75	14–15	1.06	1.42
6–8	1.02	0.59	17–19	1.06	0.81
6–9	1.19	1.74	18–20	1.07	0.91
8–9	1.11	2.17	17–18	1.01	0.00
5–7	1.09	1.87	19–20	1.02	0.00

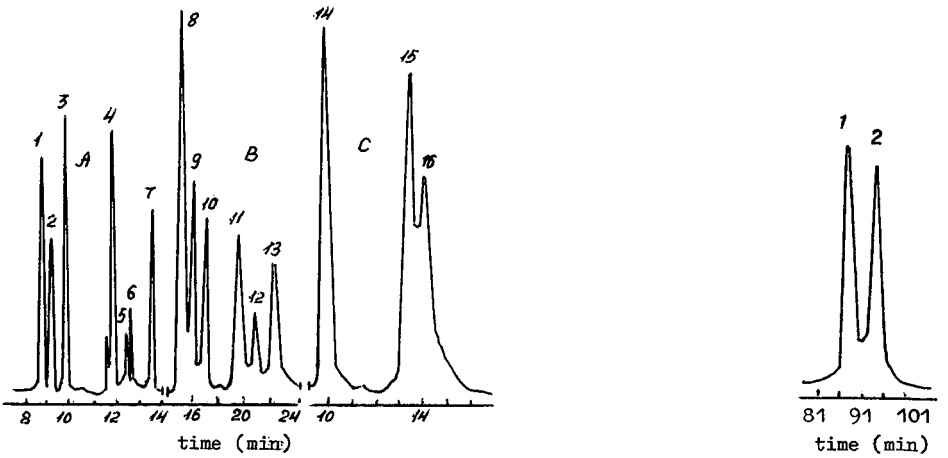


Fig. 5. Separation of insect pheromone isomers on a metal capillary column (50 m × 0.25 mm I.D.) with 1,2,3-tris(β-cyanetoxy)propane. (A) Temperature, 160°C; nitrogen flow-rate, 5.6 ml/min. (B) Temperature, 160°C; nitrogen flow-rate, 7.5 ml/min. (C) Temperature, 170°C; nitrogen flow-rate, 9.2 ml/min. For peak identification, see Table IV.

Fig. 6. Separation of (1) (*Z*)- and (2) (*E*)-7-eicosen-11-one on a glass capillary column (50 m × 0.25 mm I.D.) coated with CMB. Nitrogen flow-rate, 19 cm/s; column temperature, 178°C.

TABLE VI

KOVÁTS RETENTION INDICES (*I*), SELECTIVITY ($r_{A/B}$) AND RESOLUTION (R_s) OF GEOMETRICAL ISOMERS OF SOME INSECT PHEROMONES ON GLASS CAPILLARY COLUMNS

Compound	*Stationary phase*					
	CMB (178°C)			*DEGS (160°C)*		
	I	$r_{A/B}$	R_s	*I*	$r_{A/B}$	R_s
(*E*)-8-Decenyl acetate	–	1.25	1.54	1795	1.11	–
(*Z*)-8-Decenyl acetate	–			1818		
(*E*)-7-Dodecenyl acetate	1656	1.17	1.94	1943	1.04	0.99
(*Z*)-7-Dodecenyl acetate	1624			1956		
(*E*)-8-Dodecenyl acetate	1635	1.00	0.00	–	1.07	1.60
(*Z*)-8-Dodecenyl acetate	1636			–		
(*E*)-9-Dodecenyl acetate	1636	1.00	0.00	1943	1.05	0.97
(*Z*)-9-Dodecenyl acetate	1634			1955		
(*Z,E*)-5,7-Dodecadienyl acetate	1682	1.03	0.48	2102	1.03	0.62
(*E,E*)-5,7-Dodecadienyl acetate	1688	1.15	2.96	2120	1.05	1.07
(*Z,Z*)-5,7-Dodecadienyl acetate	1715	1.12	2.84	2129	1.09	2.03
(*E,Z*)-5,7-Dodecadienyl acetate	1739			2136		
(*Z,E*)-7,9-Dodecadienyl acetate	1697	1.10	1.86	2094	1.05	1.59
(*E,Z*)-7,9-Dodecadienyl acetate	1710	1.09	1.82	2105	1.03	1.92
(*Z,Z*)-7,9-Dodecadienyl acetate	1732	1.07	1.30	2113	1.04	1.57
(*E,E*)-7,9-Dodecadienyl acetate	1743			2119		
(*E*)-7-Eicosen-11-one	2179	1.07	1.62	–	–	–
(*Z*)-7-Eicosen-11-one	2165			–		

Fig. 7. Separation of geometric isomers of European grape vine moth pheromone on a capillary column (50 m × 0.25 mm I.D.) coated with CMB. Nitrogen flow-rate, 13.2 cm/s; column temperature, 178°C. 1 = (*Z*,*E*)-; 2 = (*E*,*Z*)-; 3 = (*Z*,*Z*)-; 4 = (*E*,*E*)-7,9-dodecadienyl acetate.

Fig. 8. Separation of (1) (*E*)- and (2) (*Z*)-8-dodecenyl acetate on a capillary column (50 m × 0.25 mm I.D.) coated with DEGS. Nitrogen flow-rate, 0.36 ml/min; column temperature, 160°C.

the capacity factor depends on variations in functional group, chain length and position and geometry of the double bond in the molecule. The alcohols elute after acetates and *cis* isomers after *trans* isomers on this stationary phase. The relative retention times of *trans* and *cis* isomers increase with reduction in chain length and increase in the distance between the double bond and the functional group. Chromatograms of the pheromones studied are shown in Fig. 5.

Glass capillary columns with DEGS (174 184 theoretical plates) and CMB (93 464 theoretical plates) were coated by the static method. The Kováts retention indices, selectivities and resolution of geometric isomers are summerized in Table VI. The separation properties of these columns towards geometric isomers of diunsaturated pheromones are better than those of the previous column. The temperature dependence of the efficiency and selectivity of CMB was studied. As the column temperature increased from a value corresponding to the crystalline state, the efficiency of the column and the separation of isomers continued to improve as the stationary phase approached the mesomorphic transition temperature (178°C). An increase in temperature above the transition point resulted in a decrease in column efficiency and separation. The selectivity of CMB showed no temperature dependence. Chromatograms of geometric isomers of some insect pheromones are shown in Figs. 6–8.

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Gas chromatographic determination of sterols in fat-soluble concentrates obtained from plant materials

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ABSTRACT

Sterols in plant materials were indentified by gas chromatography using silicone stationary phases (SE-30, OV-17, etc.) and also as their trimethylsilyl and acetyl derivatives. Mainly β-sitosterol was found in all concentrates, with smaller amounts of campesterol, stigmasterol, Δ^7-stigmasterol and Δ^7-avenasterol. Quantitative analysis was performed by purification by column liquid chromatography, extraction of the sterols with eluents of increasing polarity and determination on a column packed with 3% SE-30 at 250°C. Cholesterol was used as the internal standard.

INTRODUCTION

The determination of this composition of phytosterols present in fat-soluble concentrates obtained as a result of the complete utilization of plant materials is of importance for their application as sources of biologically active substances in the cosmetic and pharmaceutical industries[1–3]. Methods involving gas chromatography[2–11], high-performance liquid chromatography[2,12,13] and spectrophotometry[2,14,15] are the most widely used for the determination of sterols in plant materials. Gas chromatographic methods, with high resolution and sensitivity, are usually preferred.

The aim of this work was to study the qualitative and quantitative composition of phytosterols in fat-soluble concentrates obtained from plant materials using gas–liquid chromatography (GLC).

EXPERIMENTAL

Reagents

Different batches of fat-soluble concentrates were studied: nettle (*Urtica dioica* L.), venetian sumach (*Cotinus coggygria* Scop.), pine (*Pinus silvestria* L.) and *Stellaria media* (L.) Vill. obtained in the periode 1986–1988. Standard solutions of concentration 1 mg/ml were prepared of campesterol, stigmasterol and β-sitosterol in hexane and of cholesterol (internal standard) in hexane–diethyl ether (3:1).

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Silica gel G (Merck) was used in thin-layer chromatography (TLC) and Florisil (Fluka), Fuller's earth (Bulgaria) and bentonite (deposit near Kardjali, Bulgaria) in column chromatography.

Separation of phytosterols by thin-layer chromatography

A 0.5-g sample of concentrate was dissolved in 2 ml of hexane and an aliquot (100–200 μl) was separated on a silica gel G plate with light petroleum (b.p.40–60°C)–acetone (10:1). A standard solution of β-sitosterol or campesterol (20 μl) was used as the marker. On spraying with methanol, the sterols were revealed as a white band on the wet plate. The sterols were extracted with 3 × 15 ml of hexane–diethyl ether (3:1). A standard solution of cholesterol (1 ml) was added to the combined extracts, the mixture was evaporated to dryness, the residue was dissolved in 2.5 ml of hexane and 1 μl was subjected to GLC.

Separation of phytosterols by column chromatography

A 0.1–0.2-g sample of concentrate was dissolved in 2 ml of hexane and passed through a column (1 cm I.D.) containing 10 g of adsorbent (Florisil, bleach earth or bentonite). Elution was effected using solutions of increasing polarity: hexane (60 ml), hexane–diethyl ether (9:1) (90 ml) and hexane–diethyl ether (3:1) (90 ml). The sterols were extracted in the last solvent, 4 ml of a standard solution of cholesterol were added to eluate and the mixture was evaporated to dryness. The residue was dissolved in 10 ml of hexane and 1 μl was subjected to GLC.

GLC determination of sterols

A Pye Unicam Series 304 (Philips) gas chromatograph was used, equipped with columns (1.5 m × 4 mm I.D.) containing 3% SE-30, OV-17 or 2.5% QF-1 + 5% DC-200 as the stationary and a flame ionization detector. The column temperature was 250°C, the injection port temperature 270°C and the detector temperature 290°C and the carrier gas (nitrogen) flow-rate was 32–35 ml/min.

The trimethylsilyl ethers (TMSE) and the acetyl esters (AE) of the sterols were prepared[4,6,10] and were chromatographed under the same conditions.

RESULTS AND DISCUSSION

The fat-soluble concentrates of nettle, sumach, stellaria and pine are, in fact, concentrates of the unsaponifiables present in the raw materials. In order to determine their sterol contents the most often recommended methode are TLC and column liquid chromatography, and also electrophoresis[2,7,16]. In this work, the sterols were succesfully separated by TLC on silica gel G, using methanol and iodine vapour for detection. Fig. 1 shows a chromatogram for a nettle concentrate.

The types and amounts of phytosterols after TLC separation were determined by GLC on columns containing silicone stationary phases of different polarity : 3% SE-30, OV-17 and 2.5% QF-1 + 5% DC-200. Qualitative identification was achieved by comparing the relative retention times of campesterol, stigmasterol and β-sitosterol with respect to cholesterol (internal standard) obtained from the chromatogram of a standard mixture. Δ^5-avenasterol, Δ^7-avenasterol and Δ^7-stigmastenol were determined on the basis of data in the literature[6,8,11]. Table I gives the measured retention data.

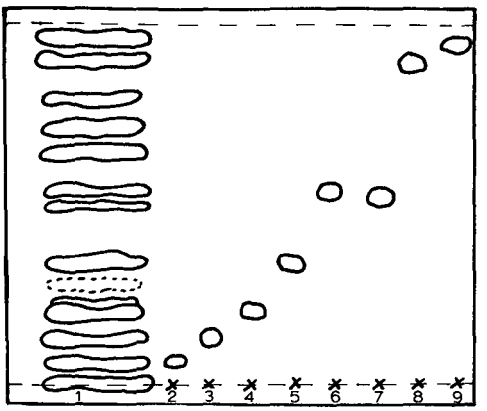

Fig. 1. TLC on silica gel G of a fat-soluble nettle concentrate using light petroleum–acetone (10:1) as the solvent and iodine vapour as spray reagent. Legend 1 = Nettle concentrate; 2 = xanthophyll of green leaves; 3 = β-sitosterol; 4 = stearyl alcohol; 5 = α-tocopherol; 6 = isopropyl stearate; 7 = isopropyl palmitate; 8 = β-carotene (from carrots); 9 = squalene.

TABLE I

RETENTION TIME (t_r') AND RELATIVE RETENTION TIMES WITH RESPECT TO CHOLESTEROL (r) OF FREE STEROLS IN NETTLE CONCENTRATE OBTAINED ON DIFFERENT STATIONARY PHASES.

Sterols	*3% SE-30*		*OV-17*		*2.5% F-1 + 5% DC-200*	
	t_r' *(min)*	r	t_r' *(min)*	r	t_r' *(min)*	r
Cholesterol	16.05	1.00	20.68	1.00	16.50	1.00
Campesterol	20.81	1.30	26.50	1.28	21.39	1.30
Stigmasterol	21.82	1.36	28.55	1.38	22.40	1.36
β-Sitosterol	25.70	1.58	32.68	1.58	25.40	1.54
Δ^7-Stigmastenol	29.10	1.82	40.12	1.94	29.70	1.80
Δ^7-Avenasterol	34.35	2.14	45.08	2.18	33.00	2.00

TABLE II

RETENTION TIMES (t_r') AND RELATIVE RETENTION TIMES WITH RESPECT TO CHOLESTEROL (r) OF FREE STEROLS AND THEIR DERIVATIVES IN NETTLE CONCENTRATES ON 3% SE-30 STATIONARY PHASE

Sterols	*Free*		*Trimethylsilyl ethers*		*Acetyl esters*	
	t_r' *(min)*	r	t_r' *(min)*	r	t_r' *(min)*	r
Cholesterol	16.05	1.00	20.05	1.00	24.18	1.00
Campesterol	20.18	1.30	25.25	1.26	31.45	1.30
Stigmasterol	21.82	1.36	27.46	1.37	32.88	1.36
β-Sitosterol	25.70	1.58	31.00	1.55	38.20	1.58
Δ^7-Stigmastenol	29.10	1.82	35.29	1.76	43.70	1.81
Δ^7-Avenasterol	34.56	2.15	43.90	2.19	51.50	2.13

TABLE III

RECOVERY OF β-SITOSTEROL AFTER TLC AND COLUMN LIQUID CHROMATOGRAPHIC (LC) SEPARATION

Raw material	*β-Sitosterol (%)*						*Recovery (%) after*	
	Determined after		*Expected quantity after 2% addition*		*Found after*			
	TLC	*LC*	*TLC*	*LC*	*TLC*	*LC*	*TLC*	*LC*
Nettles								
1	2.85	3.14	4.85	5.14	4.10	4.84	84.54	94.16
2	3.24	3.58	5.24	5.58	4.58	5.30	87.40	94.98
3	4.16	4.30	6.16	6.30	5.25	6.05	85.23	96.03
Sumach								
1	3.20	3.55	5.20	5.55	4.33	5.20	83.27	93.69
2	4.28	4.84	6.28	6.84	5.36	6.52	85.35	95.32

Trimethylsilylated and acetylated phytosterols were chromatographed on 3% SE-30 (Table II). Column liquid chromatography was also used for the separation of the sterols. The best results were achieved on columns containing Florisil, and were also good with Fuller's earth and bentonite. The most succesful fractional elution of the different concentrates was achieved with the above mentioned combination of solvents of increasing polarity.

The contents of phytosterols in concentrates from nettle and sumach are presented in Table III. They show that the analytical recovery of sterols after TLC is about 85% and after column liquid chromatography about 95%. For this reason a combination of column liquid chromatography and GLC was used for the determina-

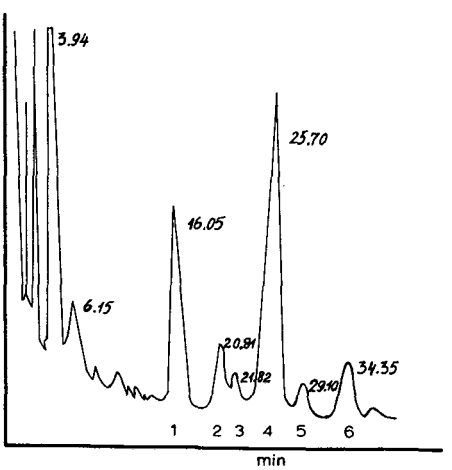

Fig. 2. Gas chromatogram of phytosterols in nettle concentrate obtained after column liquid chromatography on Florisil and GLC on an 3% SE-30 column (1.5 m × 4 mm I.D.). 1 = Cholesterol; 2 = campesterol; 3 = stigmasterol; 4 = β-sitosterol; 5 = Δ^7-stigmastenol; 6 = Δ^7-avenasterol.

TABLE IV

CONTENT OF PHYTOSTEROLS IN FAT-SOLUBLE CONCENTRATES DETERMINED BY LIQUID CHROMATOGRAPHIC SEPARATION GLC

Raw material	*Phytosterols (%)*					
	Campesterol	*Stigmasterol*	*β-Sitosterol*	*Δ^7-Stigmastenol*	*Δ^7-Avenasterol*	*Total*
Nettles						
1	0.40	0.24	2.19	Trace	0.40	3.23
2	0.40	0.24	2.50	Trace	0.42	3.66
3	0.60	0.35	0.03	Trace	0.42	4.40
4	0.60	0.20	3.14	Trace	0.50	4.44
5	0.70	0.30	4.55	Trace	0.50	6.05
Sumach						
1	0.40	0.26	2.50	Trace	0.20	3.36
2	0.64	0.28	4.20	Trace	0.25	5.37
3	0.64	0.28	3.55	Trace	0.25	4.72
4	0.67	0.30	4.84	Trace	0.25	6.06
5	0.65	0.25	5.26	Trace	0.40	6.56
Stellaria						
1	0.10	Trace	0.60	–	Trace	0.70
2	0.25	0.10	1.20	–	Trace	1.55
3	0.20	Trace	0.82	–	Trace	1.02
Pine						
1	0.20	Trace	0.58	–	Trace	0.78
2	0.20	Trace	0.60	–	Trace	0.80
3	0.25	Trace	1.50	–	Trace	1.75

tion of sterols in the different fat-soluble concentrates, despite the longer time required. Fig. 2 shows a chromatogram of the sterols in a concentrate of nettle.

Results for the contents of sterols in the different types of concentrates are given in Table IV. They indicate that the nettle and sumach concentrates are richer in sterols than those of stellaria and pine. The major sterol in all samples is β-sitosterol, its concentration varying from 67.8 to 75.2% in nettle, from 74.4 tot 80.2% in sumach, from 77.4 to 85.7% in stellaria and from 74.4 tot 85.7% in pine concentrates. The total concentration of the other sterols (campesterol, stigmasterol, Δ^7-avenasterol and Δ^7-stigmastenol) is between 14.3 and 32.2% of the total quantity of sterols.

CONCLUSIONS

A method has been developed for the determination of phytosterols in fat-soluble concentrates obtained from different plants, involving purification by column liquid chromatography and subsequent GLC determination with a recovery of about 95%. A relatively high concentration of phytosterols was found in concentrates from nettle and sumach (3.2–6.6%), which justifies their use as sterol sources for the production of various cosmetic and pharmaceutical preparations.

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Note

Sorbents with cross-linked polysiloxane phases for gas–liquid chromatography with packed columns

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Immobilized or cross-linked stationary liquid phases (SLPs) are widely used in capillary chromatography and the chromatographic parameters of immobilized SLPs have been studied in detail[1–6]. Capillary open-tubular columns with immobilized SLPs permit numerous important problems to be solved. These capillary columns are widely used in routine analysis, and their development and improvement are continuing[7–10].

SLP immobilization improves the column operating conditions, increases the SLP film stability, enhances the thermal stability of the SLP used and produces SLP films that are non-extractable with organic solvents. The last condition allows the on-column injection of large liquid samples to be performed.

We considered it of interest to apply the SLP immobilization developed in capillary gas chromatography (GC) to GC with packed columns. The first attempt to produce packed columns with immobilized phases was made by Ghaoui *et al.*[11]. They succeeded in cross-linking SE-54 on Chromosorb and the sorbent obtained showed higher thermal stability, lower bleeding and better base line stability.

Subsequently immobilization of SE-30 was described[12]. Both peroxide and γ-radiation were used for phase immobilization. Ghysen *et al.*[13] reported the immobilization of OV-101 and OV-3 on the macroporous silica Spherosil XOC 005. More recent work demonstrates the continuing interest in this problem. Sorbents with immobilized SLPs had been successfully used in high-performance gas chromatography with micropacked columns[14]. Immobilization by means of γ-radiation[15,16] and ozonolysis[17,18] has also been studied. Experimental comparison of both cross-linked and non-cross-linked phases has been carried out[19], confirming the advantages of the former.

The purpose of this work was to study the influence of the cross-linking procedure used to immobilize an SLP coated on a diatomite support on the efficiency, polarity and thermal stability of the sorbent and to investigate the distribution of the SLP on the solid support, as diatomite supports are the most widely used in GC with packed columns.

[a] Author deceased.

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EXPERIMENTAL

The diatomite supports Inerton AW and Inerton AW-HMDS (Czechoslovakia) and Dinochrom N (U.S.S.R.) were used as solid supports. Siloxane gums, namely the non-polar polydimethylsiloxane SE-30 (Merck, F.R.G.) and the polar poly(dimethyl-fluoropropyl)siloxane SKTFT-50X (U.S.S.R.), were used as SLPs. The solid support was coated from the SLP solution as described elsewhere[20]. Siloxane gums were immobilized by means of either benzoyl peroxide or γ-radiation (^{60}Co, 300 K). When the peroxide method was used the solid support was coated simultaneously with 3% of the cross-linking agent benzoyl peroxide (with respect to the SLP mass). After removal of the solvent, immobilization was carried out by slow heating to 200°C (2–4 h) in an inert gas flow, immobilization taking place in a chromatographic column.

To immobilize the sorbent by means of radiation, the sorbent was radiated with doses of 2–10 Mrad. After cross-linking, the residual non-immobilized phase was extracted directly in the column by passing an appropriate solvent (ethyl acetate, toluene or benzene) for 15–20 h.

An LKhM-8MD Model 5 gas chromatograph (U.S.S.R.) with a flame-ionization detector was used. The carrier gas was nitrogen or helium.

To evaluate the sorbent surface structure and distribution of SLP on the surface, scanning electron microscopy was used. A JSM-50A scanning electron microscope (Jeol, Japan) with an accelerating electron probe voltage of 25 kV under secondary electron emission was used. Sorbent surfaces were studied at magnifications of 1000 and 3000, corresponding to the surface areas of 10 000 and 300 μm^2.

To estimate the immobilization yield, capacity factor (k') with respect to *n*-alkanes were determined before and after extraction. The thermal stability of the SLPs was characterized by the background value at 280°C. This value was determined as the baseline deviation with the carrier gas flow switched off.

Retention indices with respect to benzene, butanol, 2-pentanone, 1-nitro-propane and pyridine were determined to evaluate the polarity of the SLPs.

The efficiency of analytical and micropacked columns was determined as the minimum height equivalent to a theoretical plate (H_{min}). The H_{min} value was determined experimentally from the Van Deemter equation (with respect to *n*-alkanes at several temperatures).

RESULTS AND DISCUSSION

The results of the comparative study of sorbents with cross-linked and non-cross-linked SLPs are given in Table I. The immobilization yield varied from 60 to 100% depending on the cross-linking conditions The immobilization efficiency depends not only on the cross-linking conditions (temperature, amount of cross-linking agent), but also on the sorbent surface, in particular on the impurities present.

Cross-linking hardly influenced the sorbent efficiency, especially in the optimum range (Fig. 1). It can be noted that at carrier gas flow-rates higher than the optimum, sorbents cross-linked under radiation were less effective than those non-immobilized or cross-linked with peroxide (Table I). This phenomenon was probably due to the greater number of cross-linking sites between the SLP macromolecules. An increase in the radiation dose did not lead to a higher immobilization yield. The same effect has been noted with open-tubular columns[21].

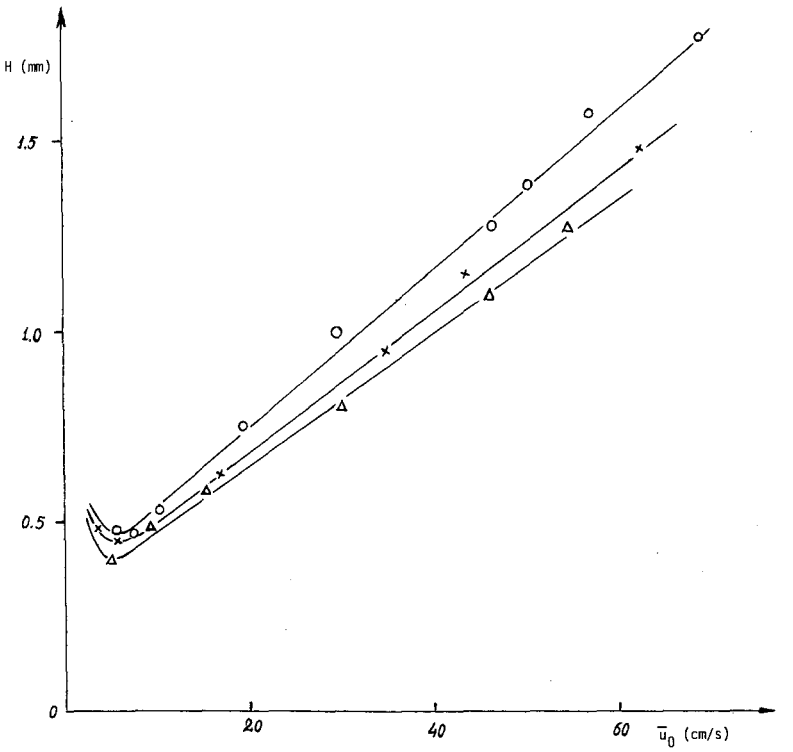

Fig. 1. Van Deemter dependences with respect to *n*-decane for the column packed with Inerton AW coated with immobilized and non-immobilized SE-30 at 80°C. ○ = Cross-linked with γ-radiation; △ = cross-linked with benzoyl peroxide; × = non-cross-linked. Fused-silica column, 57 cm × 1 mm I.D. $\bar{u}_0$ is the carrier gas average linear rate corrected in accordance with the gas compressibility.

After immobilization the thermal stability of the sorbent was increased. Therefore, cross-linked phases can be used at high temperatures without any baseline deterioration. Polarity changes for both phases (SE-30 and SKTFT-50X) as a result of immobilization were also studied. Table II shows the retention indices for five compounds used to determine McReynolds constants. Immobilization caused virtually no change in the SLP polarity. An increase in the retention indices took place with SE-30 (irrespective of the cross-linking procedure). With SKTFT-50X γ-radiation caused a decreased in the retention indices and treatment with peroxide led to an increase.

The distribution of cross-linked SLPs over the surface after removal of non-immobilized residues was studied by scanning electron microscopy. Fig. 2 shows that immobilized SLPs provide a uniform cover on the sorbent surface. When γ-radiation was used (Fig. 2b) the SLP film was thinner than that obtained with peroxide (Fig. 2a). This is in agreement with chromatographic data on capacity factors. It should also be mentioned that there were no microcracks on the cross-linked SLP films, in contrast to non-immobilized SLPs[22]. The data obtained are in a good agreement with those for open-tubular capillary columns[21].

We tried to wash off the cross-linked SLPs by repeated sample injection into the

TABLE I

CHROMATOGRAPHIC PARAMETERS OF SORBENTS WITH POLYSILOXANE SLPs BOTH CROSS-LINKED AND NON-CROSS-LINKED ON THE SURFACES OF DIATOMITE SOLID SUPPORTS

Column dimensions (length × I.D.) and material	*SLP (%); solid support*	*Cross-linking method*	*Yield (%)*	*Column temperature (°C)*	*Capacity factor, k'*	H_{min} *(mm)*	*Column background*[a] *at 280°C*	*Mass-transfer coefficient, C (s)*
69 cm × 0.3 mm, fused silica	3% SE-30, Dinochrom N, 0.06–0.08 mm	–	–	100	30.2, n-C_{12}	0.29	–	$3.8 \cdot 10^{-4}$
		Peroxide	79	100	23.7, n-C_{12}	0.34	–	$4.6 \cdot 10^{-4}$
		γ-Radiation	90	100	27.0, n-C_{12}	0.40	–	$18.0 \cdot 10^{-4}$
57 cm × 1 mm, stainless steel	5% SE-30, Inerton AW 0.06–0.1 mm	–	–	80	24.8, n-C_{10}	0.45	–	$19.0 \cdot 10^{-4}$
		Peroxide	86	80	21.3, n-C_{10}	0.39	–	$16.0 \cdot 10^{-4}$
		γ-Radiation	86	80	21.4, n-C_{10}	0.47	–	$23.0 \cdot 10^{-4}$
100 cm × 3 mm, stainless steel	5% SKTFT-50X, Dinochrom N, 0.1–0.2 mm	–	–	120	10.4, n-C_{12}	0.99	–	$33.0 \cdot 10^{-4}$
		Peroxide	79	120	8.4, n-C_{12}	1.10	–	$7.2 \cdot 10^{-4}$
200 cm × 3 mm, stainless steel	2.5% SE-30, Inerton AW HMDS, 0.2–0.25 mm	–	–	120	9.1, n-C_{12}	0.87	$1.24 \cdot 10^{-10}$	–
		Peroxide	82	120	7.5, n-C_{12}	0.86	$0.70 \cdot 10^{-10}$	–
		γ-Radiation	82	120	7.5, n-C_{12}	1.08	$0.40 \cdot 10^{-10}$	–

[a] Column background is a deviation of the recorder pen with the carrier gas switched off.

TABLE II

RETENTION INDICES OF POLAR COMPOUNDS FOR SORBENTS WITH SILICONE SLPs CROSS-LINKED AND NON-CROSS-LINKED ON THE SURFACE OF INERTON AW AT 120°C

SLP type[a]	*Retention index*					
	Benzene	*1-Butanol*	*2-Pentanone*	*1-Nitropropane*	*Pyridine*	*Total*
SE-30, non-cross-linked	672	650	681	722	877	3602
SE-30, cross-linked with γ-radiation, washed	668	647	672	719	865	3571
SE-30, cross-linked with peroxide, washed	671	646	674	720	861	3572
SKTFT-50X, non-cross-linked	731	740	848	937	1026	4282
SKTFT-50X, cross-linked, with peroxide, washed	740	735	851	938	1049	4313
SKTFT-50X, cross-linked with γ-radiation, washed	727	729	841	938	1026	4261

[a] When Inerton AW was used, 10% (mass) SLP was coated. With of SE-30 and SKTFT-50X the cross-linking yield with benzoyl peroxide was 98 and 90%, respectively, and with γ-radiation 84 and 79%, respectively.

column. Samples of 100–1000 μl were injected into the column (2 m × 3 mm I.D.) at temperatures near the solvent boiling point. There was no phase removal, the capacity factors and column efficiency remaining unchanged. When five samples (200 μl each) were injected into a column packed with non-immobilized SLP, the column parameters deteriorated irreversibly. It seems expedient to use cross-linked SLPs for preparative chromatography because it is well known that the SLP is often washed off the initial part of a preparative column.

Hence, sorbents with cross-linked phases can be used for trace analysis by injecting large samples, microimpurities being concentrated directly in the column. This is the most promising application of the sorbents discussed.

CONCLUSION

Sorbents based on SLPs cross-linked by means of peroxide or γ-radiation on a diatomite support and non-cross-linked SLPs are characterized by similar efficiency, polarity and distribution over the solid support, possessing all the advantages of cross-linked phases.

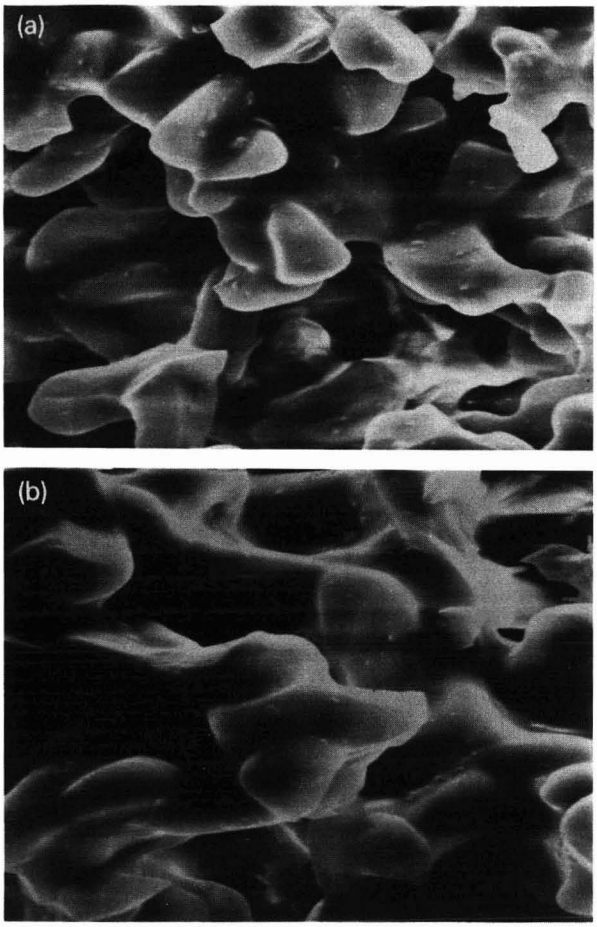

Fig. 2. Microsection of Interton AW surface coated with dimethylsilicone gum SE-30 cross-linked with (a) γ-radiation and (b) benzoyl peroxide. Magnification × 1000.

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CHROM. 22 155

High-resolution gas chromatography with liquid crystal glass capillaries

XI[a]. Separation of isomeric C_8 and C_9 hydrocarbons

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ABSTRACT

Pyrimidine mesogenic stationary phases were investigated for the separation of geometric and positional isomers and diastereomers of hydrocarbons. Owing to the high selectivity of the stationary phase, combined with a suitable mesogenic temperature range and the efficiency of capillary columns, enhanced separations of C_8 and C_9 isomeric alkanes, alkenes and alkadienes could be achieved.

INTRODUCTION

The separation of lower boiling isomeric hydrocarbons using liquid crystals as stationary phases in capillary gas chromatography demands special requirements. Previously investigated selective liquid crystalline phases had too high temperatures of the mesogenic range, and some phases with a low mesogenic temperature range had too low selectivities for the separation of isomeric hydrocarbons. The highest selectivity of the liquid crystal was found on 4-methoxy-4′-ethoxyazoxybenzene (MEAB)[1,2], but its mesophase range is too high for the efficient separation of lower boiling hydrocarbons. Another liquid crystal, 4-*n*-pentylacetophenone-(O-4-*n*-pentyloxybenzoyl oxime) (PBO)[3], has a low mesogenic temperature range, but its selectivity with respect to the present separation problem is low.

The aim of this work was to test new pyrimidine mesophases combining high selectivity, mesogenic range at low temperatures and a high efficiency of the capillary column for the separation of isomeric C_8 and C_9 hydrocarbons.

[a] For Part X, see ref. 10.

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EXPERIMENTAL

The separations were carried out on 4-*n*-pentylbenzoic acid 4′,5-*n*-hexylpyrimidine-2-ylphenyl ester (PBHP) having a mesophase range of 37–159°C and possible supercooling to about 25°C[4,5], and on 4-*n*-propylbenzoic acid 4′,5-*n*-hexylpyrimidine-2-ylphenyl ester (PrBHP) having a mesophase range of 65–170°C and possible supercooling to about 28°C. After etching with gaseous hydrogen chloride, the glass capillaries were coated dynamically with a 5% (w/w) solution of liquid crystal in chloroform. The first capillary with PBHP (82 m × 0.25 mm I.D.) had an efficiency of 210 000 theoretical and 103 000 effective plates at $k = 3.1$ for *trans*-3-nonene at 33°C. The second capillary with PrBHP as the stationary phase (112 m × 0.11 mm I.D.) had an efficiency of 306 000 theoretical and 130 000 effective plates at $k = 1.83$ for 3,5-dimethylheptane at 40°C.

The phase behaviour and selectivity of the mesophase were measured using a glass column (3 m × 3 mm I.D.) packed with 10% (w/w) of stationary phase on Chromosorb W HP.

All possible isomeric *n*-nonenes and *n*-nonane, the isomeric *n*-octadienes with conjugated double bonds (isomerization product of the α,ω-octadiene) and the diastereomeric 3,4-dimethylhexanes and 3,5-dimethylheptanes served as model mixtures.

RESULTS AND DISCUSSION

Systems for the separation of lower boiling hydrocarbons should have good sorption qualities, high efficiences and high selectivity combined with a suitable temperature of the mesogenic range. The mesogenic pyrimidine compounds mentioned above fulfilled all these requirements.

Fig. 1 shows the dependence of the specific retention volume, V_g^0, of *n*-octane on the reciprocal temperature measured on a packed column[6], and demonstrates that the nematic mesophase range can be extended to about 25°C by supercooling.

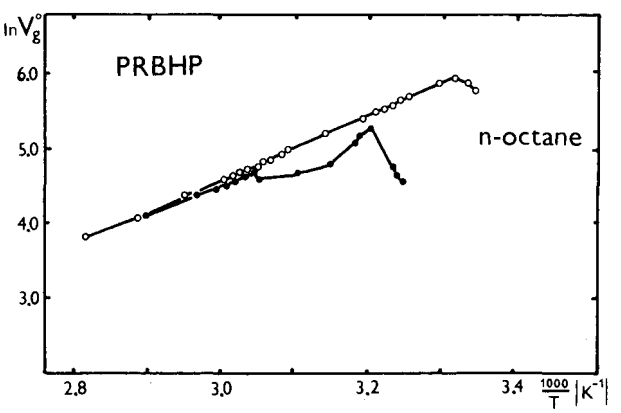

Fig. 1. Dependence of the specific retention volume of *n*-octane on the reciprocal temperature on PBHP. ● = Heating; ○ = cooling.

TABLE I

SELECTIVITY FACTORS, α, AND MESOPHASE RANGES OF DIFFERENT LIQUID CRYSTALS

Liquid crystal	$\alpha^{50°}_{p\text{-}/m\text{-}X}$	*Mesophase range (°C)*
MEAB	1.20[a]	70–150
PrBHP	1.16	28–170
PBHP	1.15	25–155
PBO	1.07	35–95

[a] In the capillary column with a thick film of stationary phase, supercooling to 42°C was achieved[2].

Table I gives the selectivity factors, α, for *p*- and *m*-xylene at 50°C and mesogenic ranges for MEAB, PrBHP, PBHP and PBO liquid crystal. Just above the melting point, the selectivity factor for PBHP is 1.13, reaching a value of 1.20 on supercooling to 25°C. In comparison, MEAB can usually be supercooled only to 70°C with a corresponding α-value of 1,16[2]; this demonstrates that PBHP, with a favourable temperature of the mesophase range and with a selectivity comparable to that of MEAB, is more suitable for the separation of lower boiling hydrocarbons which require a lower temperature of the separation column.

The contribution of mesophase selectivity to the separation of lower boiling hydrocarbons with similar physico-chemical properties is relative small and requires its combination with the high efficiency of capillary columns for optimum separations. The pyrimidine phases permit a good coating in glass capillaries, as shown by an efficiency of more than 100 000 effective plates for C_8 and C_9 hydrocarbons.

The performance of these systems for the separation of positional, geometric and diastereomeric C_8 and C_9 hydrocarbons with similar physico-chemical properties is illustrated by the following examples.

Separation of isomeric n-nonenes

Fig. 2 shows the separation of all isomeric *n*-nonenes and *n*-nonane. With the

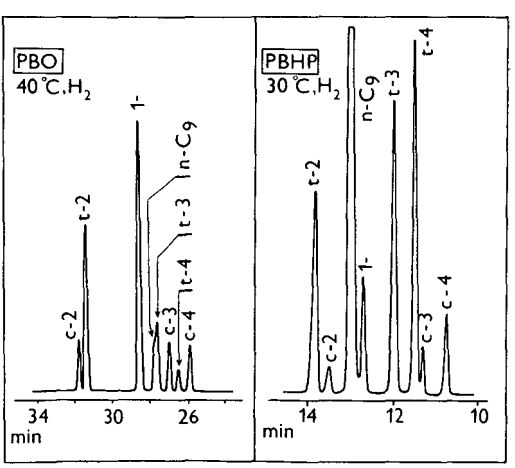

Fig. 2. Separation of isomeric *n*-nonenes and *n*-nonane on PBO and PBHP. t = *trans;* c = *cis.*

TABLE II

RELATIVE RETENTIONS OF POSITIONAL AND GEOMETRIC ISOMERIC *n*-NONENES ON PBO AND PBHP AT 40°C

Pair of n-nonenes	*Relative retention*	
	PBO	*PBHP*
trans-2-–*trans*-3-	1.174	1.206
trans-3-–*trans*-4-	1.057	1.051
cis-2-–*cis*-3-	1.229	1.257
cis-3-–*cis*-4-	1.055	1.078
trans-2-–*cis*-2-	0.985	1.020
trans-3-–*cis*-3-	1.031	1.063
trans-4-–*cis*-4-	1.029	1.091

non-mesogenic stationary phases, incomplete separations with long separation times were obtained, in spite of the high efficiency, which could be improved by using the mesogenic stationary phase PBO[7]. The complete separation of all *n*-nonene isomers and *n*-nonane succeeded with PBHP despite the lower separation efficiency in comparison with the PBO column. Whereas on the mesogenic system with PBO, having a separation efficiency of 280 000 effective plates, the separation of these isomers is possible in 34 min, the separation system using more selective mesophase PBHP achieved an enhanced separation in about 14 min. This 2.5-fold faster separation is attributed to the higher mesophase selectivity for positional and geometric isomeric alkenes and the related more effective temperature optimization of the separation.

The higher isomeric selectivity of PBHP in comparison with PBO is obvious considering the change in the retention sequence of the pairs of *cis*-3-–*trans*-4- and *cis*-2-–*trans*-2-nonene isomers as the *trans* isomers have higher retention values (Table II). It also finds expression in the greater retention of *n*-nonane than 1-nonene, altough the polarity of PBHP is higher than that of PBO. In both instances the measurements were carried out at the optimized temperatures and a temperature difference of 10°C gave no change in the retention sequence.

Table II also shows the lower relative retention of the *trans*-3-–*trans*-4-nonene pair on PBHP in comparison with those on PBO. We observed a similar effect with the *trans*-5-–*trans*-6-tridecene pair[8]. This similar situation found for *trans*-*n*-alkenes with an odd number of carbon atom and the double bond in the middle of the carbon chain is observed after a change in the carbon number in the chain by four. This is connected with the change in the orientation of the carbon chain ends for both isomers when the double bond is shifted within the carbon chain.

Separation of isomeric n-octadienes

In Fig. 3, the separation of all isomeric conjugated *n*-octadienes on the PBHP capillary is compared with the separation on a 200-m squalane capillary column (300 000 effective plates)[9]. Despite the high efficiency of the squalane column, three pairs of isomers could no be separated, whereas the use of the three times less effective PBHP capillary led to a baseline separation of all conjugated *n*-octadiene isomers, even with a shorter analysis time.

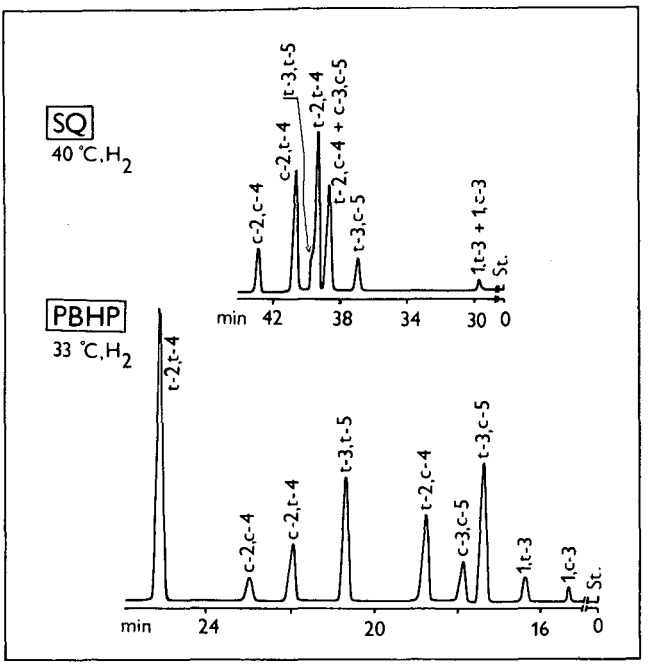

Fig. 3. Separation of isomeric conjugated *n*-octadienes on squalane and PBHP.

The retention sequence of *n*-octadiene isomers on PBHP differs from that on squalane. The peaks were identified on the basis of structure–retention correlations; for 1,*trans*-3-, *trans*-2, *trans*-4- and *trans*-3,*trans*-5-octadienes it was confirmed by the reaction with maleic anhydride[9]. The high selectivity of PBHP is mostly obvious in the increase in the retention of *trans*-2,*trans*-4-octadiene, which is eluated as the last component of the mixture, and in the separation of 1,*trans*-3-octadiene after the 1,*cis*-3- isomer.

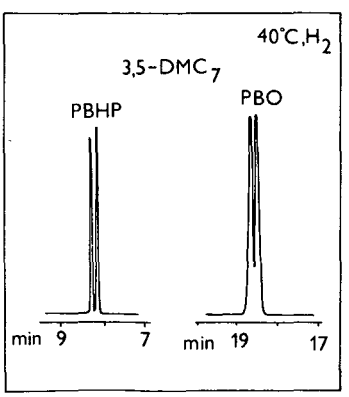

Fig. 4. Separation of diastereomeric 3.5-dimethylheptanes on PBO and PBHP.

TABLE III

RETENTION INDICES OF DIASTEREOMERIC 3,5-DIMETHYLHEPTANES ON PBO AND PBHP AT 40°C

Liquid crystal	*Retention index of diastereomeric 3,5-dimethylheptanes*	
PBO	811.9	813.4
PBHP	792.0	795.3

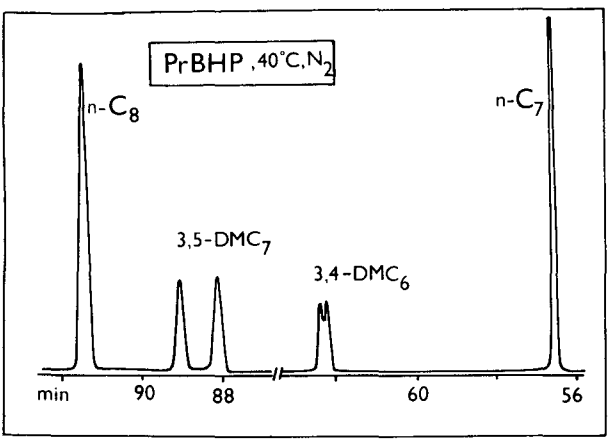

Fig. 5. Separation of diastereomeric 3.4-dimethylhexane and 3.5-dimethylheptane on PrBHP.

TABLE IV

DIFFERENCE IN RETENTION INDICES, δI, FOR DIASTEREOMERIC C_8–C_{10} ALKANES ON THE DIFFERENT STATIONARY PHASES AT 40°C

Diastereomeric alkane	δI				
	Squalane[b]	*Ucon LB*[b]	*SE*[b,c]	*PBO*	*PBHP*
3.4-Dimethylhexane	0.0	0.0	0.3	0.0	0.9
3.5-Dimethylheptane	0.0	0.0	1.1	1.5	3.3
3.6-Dimethyloctane	0.0	0.0	0.8	1.1	2.4[a]

[a] Hypothetical value.
[b] See ref. 10.
[c] Silicones similar to SE-30 and SE-54.

Separation of diastereomeric C_8–C_9 alkanes

In a previous study[10], the separation of diastereomeric C_8–C_{10} alkanes on mesogenic PBO phase was investigated. Fig. 4 compares the separation of diastereomeric 3,5-dimethylheptanes on PBO and PBHP. PBO was coated on a capillary giving 280 000 effective plates, whereas the PBHP capillary had a lower efficiency of 103 000 effective plates. Fig. 4 clearly demonstrates a better and faster separation of the diastereomers on PBHP owing to its higher selectivity. At 40°C the selectivity factor α (relative retentions) of diastereomers is 1.032 on PBHP and 1.014 on PBO. The retention index difference is 3.3 i.u. on PBHP and 1.1 i.u. on PBO. A decrease in temperature to 30°C leads to $\alpha^{PBHP} = 1.049$ and $\delta I = 4.6$ i.u. The higher selectivity of PBHP is also shown in the lower retention indices of the diastereomeric 3,5-dimetylheptanes (Table III).

A higher sorption of lower boiling hydrocarbons and hence the separation of diastereomeric 3,4-dimethylhexanes was achieved on a second column with a lower phase ratio β, based on a smaller I.D. of the capillary. The separation of diastereomeric 3,5-dimethylheptanes with this separation system is longer than that on the first column (Fig. 5).

In Table IV the differences in retention indices (δI), are given for the most difficult to separate diastereomeric C_8–C_{10} alkanes. The contribution of the liquid crystal PBHP to their separation is evident.

CONCLUSIONS

Different mesophase selectivities are show in the different retention behaviours of positional and geometric isomers and diastereomers of hydrocarbons. With the geometric isomers of aliphatic hydrocarbons a change in their retention sequence on the different types of liquid crystals was found.

The mesogenic pyrimidine compounds PBHP and PrBHP, with high selectivity, suitable mesophase range and good efficiency of glass capillary columns, permit better and faster separations of isomeric C_8–C_9 alkenes and alkadienes and of diastereomeric alkanes compared with non-mesogenic and other mesogenic stationary phases.

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CHROM. 22 209

Qualitative analysis of a commercially available phenol–formaldehyde resin, by static and dynamic headspace analysis using a mass-selective detector

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ABSTRACT

Static headspace and dynamic headspace procedures for volatile substances in solid matrices were investigated and compared by gas chromatography–mass spectrometry (GC–MS). For this purpose volatile components of a commercially available phenol–formaldehyde resin were analysed by GC–MS, first using the traditional static headspace method. A further study of the method aimed at obtaining a greater concentration, together with increased selectivity and a more reliable structural determination of the volatile substances, led to the use of the dynamic headspace (purge and trap) method. Following the latter method, after enrichment, the trap contents were recovered in solvents with different polarities in order to concentrate selectively the substances contained in the vapours of the resin itself. The solutions obtained were analysed by GC–MS.

INTRODUCTION

Volatile organic compounds in solid matrices can be analysed by gas chromatography (GC) using the static headspace and dynamic headspace (purge and trap methods)[1–3]. The former method consists in heating the solid matrix in a hermetically closed vessel. The gaseous phase is analysed after a certain periode once equilibrium has been reached. The results are thus interpreted as a description of the gas–solid system at determined pressure, temperature and volume values (analysis of the thermodynamic equilibrium system). In the dynamic headspace method, the sample is prepared using the same procedure as in the former method and is swept by an inert gas stream (helium) that conveys the gaseous volatile compounds to a cold trap usually kept at a temperature 30–40°C lower than the retention temperature of the substance to be detected. The trap is filled with a solid phase (e.g., Tenax TA, graphitized carbon, SE-30, OV-1 on Chromosorb, etc.). After rapid heating, the stripping gas from the trap is carried into a gas chromatograph for analysis.

Sometimes the gas flow required both to wash the trap thoroughly and to allow for well resolved chromatographic peaks cannot be injected directly (split or splitless)

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into the capillary column. One can then operate according to one of two procedures. In one, collect all stripping gas in a 0.53-mm empty and passivated/silanized fused-silica wide-bore capillary, about 20 cm long, kept at low temperature under liquid nitrogen. Insert the capillary tube in a split/splitless injector and heat it quickly under an inert gas flow. Adjust the analysis parameters according to the type of capillary column being used. In the other, immerse the purge and trap outlet capillary tube in a few millilitres of pure polar or apolar solvent maintained at a low temperature and allow the stripping gas to bubble in the liquid. It can be immediately understood why the latter method can only be used for qualitative analyses. Both the bubbling (small bubbles with as long a time of bubble–solvent contact as possible) and solvent evaporation phases are critical. Decreasing the final volume with respect to the initial volume would bring about a proportional increase in the impurities likely to be present in the blank.

The main advantage of the dynamic over the static headspace method is the wider range of substances that can be detected. This derives from the fact that the vial washing gas carries a greater amount of components towards the trap and thus to the detector used (non-equilibrium system analysis).

EXPERIMENTAL

The solid matrix on which tests were performed is a common phenol–formaldehyde resin. Such a resin contains a wide range of compounds obtained from the condensation reaction of phenols or substituted phenols with aldehydes. Resolic resins are obtained when operating with an excess of aldehyde and a basic catalyst (ammonia, sodium hydroxide solution), whereas novolak resins are obtained when there is an excess of phenol under acidic conditions. *para*-Alkyl-substituted phenols produce water-soluble liquid linear resins (mol. wt. 125–150) or organic solvent-soluble solid resins (mol. wt. > 1000). The use of molten resin as raw material or as a co-blender in polymeric matrices promotes irritating, corrosive and foul-smelling vapours, even if used at relatively low temperatures (80–100°C).

Apparatus and conditions

The gas chromatograph was an HP 5890 (Hewlett-Packard) with an HP 5970 mass-selective detector in the total ion current mode. The transfer line was maintained at 280°C. The inlet type was split only or splitless. The mass range was 33–350 U. The split operating mode was used. The oven temperature was programmed from 40 to 250°C at 8°C/min. The column was 12 m × 0.2 mm I.D. coated with Ultra 2 fused silica (Hewlett-Packard) (cross-linked 5% phenyl methyl silicone), with a 0.32-μm film thickness. The carrier gas was helium (SIO-Alphagaz, N60 type, purity $\geqslant$99.9999%) with a column head pressure of 50 kPa and a split vent of 60 cm^3/min. The sample was 0.2 ml (static headspace method) or 2 μl (dynamic headspace method). An HP 59970 MS Chemstation Rev. 3.2 data station installed on an HP 9000 Series 300 was used.

For the dynamic headspace (purge and trap method) a Dani SPT 37.50 equipped with a Dani constant incubation time (CIT) device was used (Fig. 1). A constant-temperature bath was maintained at 130°C (silicone oil) and the temperature of the switching valve and transfer line was 135°C. The incubation time was 2 or

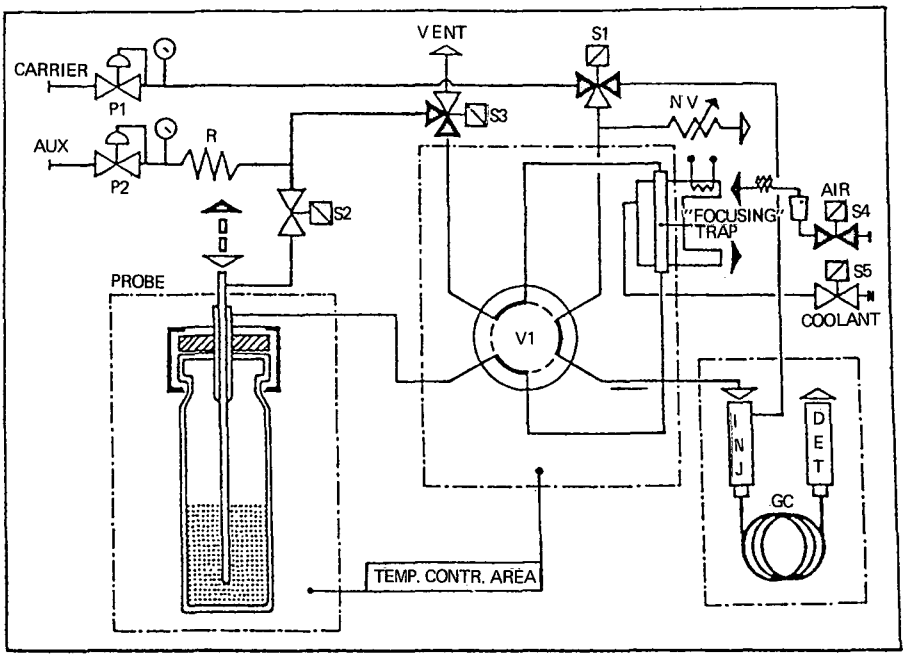

Fig. 1. Dani SPT 37.50 pneumatics scheme. P1, P2 = pressure regulators; S1–S5 = solenoid valves; NV = needle valve; R = calibrated restrictor; V1 = six-port pneumatic valve; INJ = injection: DET = detection; TEMP.CONTR. = temperature-controlled.

24 h. The trap packing was 200 mg of Tenax TA (60–80 mesh) and the trap temperature was increased from 30 to 300°C at 1200°C/min. The cooling time was 4 min, purging time 3 min, trap desorption time 2 min and trap back-flushing time 4 min (2 h after each analysis set) at 300°C.

Reagents

Methanol (HPLC grade) was obtained from Carlo Erba. A 0.01 *M* solution of sodium hydroxide in distilled water was prepared. SP 1045 resin (Schenectady Europe) (mol. wt. > 1000) was used.

RESULTS AND DISCUSSION

Static headspace analysis

Two resin samples were prepared in 40-g sealed glass containers with silicone septa. After preconditioning at 130°C (2 h for the first sample, 24 h for the second), 0.2 ml of gas phase was injected into the GC–mass spectrometry (MS) system (see Figs. 2 and 3). On comparing the two chromatograms obtained, it was found that after 24 h the following appeared: methanoic acid (Table I, peak 1), phenol (12), diethylstilbestrol (30) and 2,4-(1,1,3,3-tetramethylbutyl)diphenol (34). Further, there is a decrease in 4-(1,1,3,3-tetramethylbutyl)phenol (31). The analysis performed after conditioning for only 2 h at 130°C showed a non-equilibrium system (Fig. 3), whereas the second analysis showed a thermodynamic steady equilibrium system. In fact, after

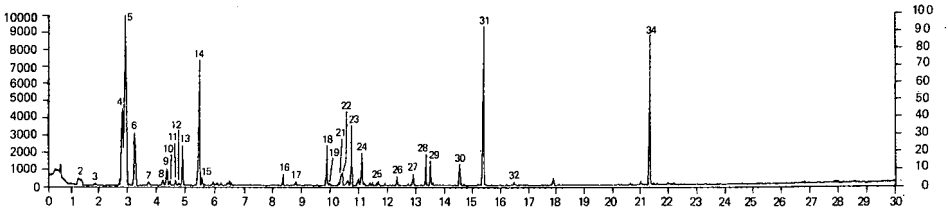

Fig. 2. Resin after 2 h at 130°C. Static headspace method.

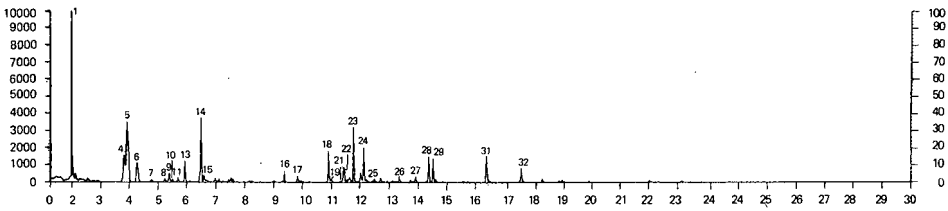

Fig. 3. Resin after 24 h at 130°C. Static headspace method.

TABLE I

IDENTIFIED PEAKS IN FIGS. 2–6

Peak No.	*Compound*[a]	*Peak No.*	*Compound*
1	Methanoic acid	20	1,2-Diaminobenzene
2	5,5-Dimethyl-2-hexene	21	4-(1,2-Dimethylethyl)phenol
3	4,4-Dimethyl-2-pentanone	22	Cyclohexane, substituted
4	Ethylbenzene	23	1-Propene-2-methyl-, tetramer
5	1,3-Dimethylbenzene + 1,4-dimethylbenzene	24	1-Propene-2-methyl-, tetramer
6	1,2-Dimethylbenzene	25	2,5-Bis(1-methylethyl)phenol
7	(1-Methylethyl)benzene or isopropylbenzene	26	4,4-Dimethyl-1-ethoxy-2-pentene
8	1-Methyl-*x*-ethylbenzene	27	Heptyl hexyl ether
9	1-Methyl-*x'*-ethylbenzene	28	1-Ethyl-*x*-(1,1,3,3-tetramethylbutyl)benzene
10	1-Methyl-*x''*-ethylbenzene	29	1-Ethyl-*x'*-(1,1,3,3-tetramethylbutyl)benzene
11	1,2,4-Trimethylbenzene	30	Diethylstilbestrol
12	Phenol	31	4-(1,1,3,3-Tetramethylbutyl)phenol
13	1,2,3-Trimethylbenzene	32	2-Ethyl-4-(1,1,3,3-tetramethylbutyl)phenol
14	1-Methyl-3-(1-methylethyl)benzene	33	1,2-Benzenedicarboxylic acid, bis(2-methoxyethyl) ester
15	2-Ethyl-1-hexanol	34	2,4-(1,1,3,3-Tetramethylbutyl)diphenol
16	1,4-Bis(1-methylethyl)benzene	35	4,4'-Oxydianiline
17	1-(1,1-Dimethylethyl)-3,5-dimethylbenzene	36	[1,1':3',1''-Terphenyl]-2'-ol
18	1,1,3,5-Tetramethylcyclohexane	37	[1,1'-Phenoxy:3',1''-bisphenyl]-2'-ol
19	4-(1,1-Dimethylethyl)phenol	*	Column bleeding

[a] x = Uncertain positions of substituents ($x \neq x' \neq x''$)

40 h, the same results were also achieved and a similar chromatogram was obtained (not shown).

Dynamic headspace analysis

Two sets of phenolic resin samples (three vials for each) were prepared with a total weight of 40 g per set. Preconditioning was performed inside the Dani SPT 37.50 instrument in a suitable silicone-oil bath maintained at a 130°C for 2 and 24 h for the first and second set of samples, respectively. After the conventional purging and trapping phases, the washing gas for each vial was allowed to bubble into 0.5 ml of methanol kept in a Dewar vessel containing solid carbon dioxide. After each set of three vials, the trap was left to backflush at 300°C for 2 h. A 2-μl aliquot of the collecting vessel contents was injected into the GC–MS system (see Figs. 4 and 5).

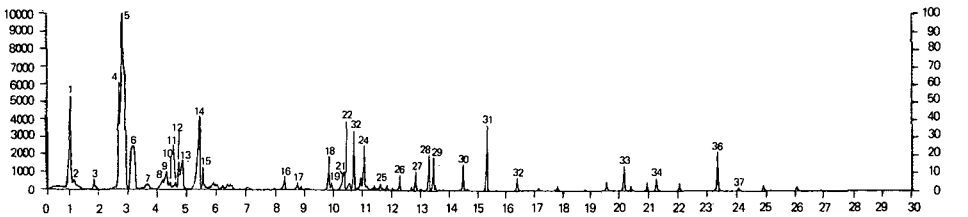

Fig. 4. Resin after 2 h at 130°C. Dynamic headspace method (methanol).

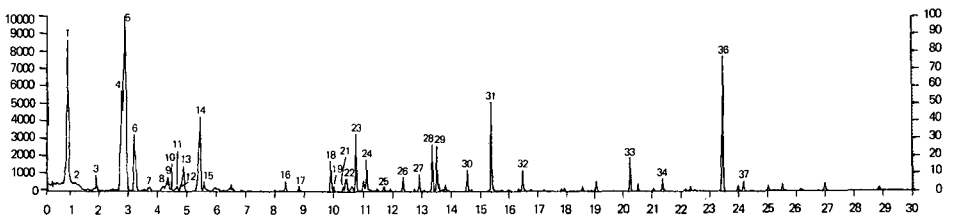

Fig. 5. Resin after 24 h at 130°C. Dynamic headspace method (methanol).

Interpretation of the mass spectra of the peaks allowed an approximate identification of the resin components. However, in many instances the exact identification of a component proved difficult owing both to an insufficient GC selectivity and to the fact that the amount involved was too small to give an easily identifiable mass spectrum. A new set of samples was therefore prepared. After their preconditioning at 130°C for 24 h, the trap eluate was collected in 0.5 ml of 0.01 *M* sodium hydroxide solution kept at 0°C in an ice–water bath. In this manner the substituted phenols (main responsible for the imperfect peak splitting) remained in solution as anions in equilibrium with the uncharged form. A slight helium flow for a few minutes was sufficient to remove the traces of hydrocarbons and insoluble apolar components. A chromatogram containing peaks almost all of which are due to polar compounds (see Fig. 6) and comparison of the chromatograms shown in Figs. 2–6 to one another, made it possible to identify 38 different compounds (see Table I). As described in the Introduction the two chromatograms in Figs. 4 and 5 clearly represent a non-equilib-

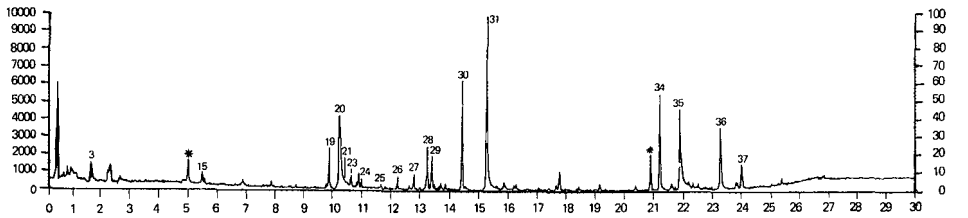

Fig. 6. Resin after 24 h at 130°C. Dynamic headspace method (water).

rium system. The only difference, probably ascribable to slower desorption kinetics from the molten matrix, is a marked increase in some heavy fractions (peaks 36 and 37). Fig. 6 shows two peaks that were not observed in the previous analyses, *i.e.*, 20 and 35. These amine compounds are probably due to the type of catalyst used in the resin synthesis *i.e.*, ammonia or an organic amine.

CONCLUSIONS

Upgrading various operating parameters makes it possible to determine most impurities in a solid matrix. Obviously, a more in-depth characterization could provide a broader picture. To achieve such detail one must operate with traps filled with different phases (*e.g.*, Carbotrap) kept at low temperatures (liquid nitrogen cooling), directly inject the purge and trap eluate into the capillary column or change the collecting solvents. The operating conditions can thus be optimized as a function of the compound or compound class involved.

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CHROM. 22 153

Note

Headspace analysis of polar compounds in air

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Gaseous effluents in the detergent industry contain volatile organic substances such as C_1–C_4 aliphatic alcohols, ketones and ethers, the approximate safety level of which in the atmosphere is 0.1 mg m^{-3}. Although some gas chromatographic (GC) methods have been developed for the determination of atmospheric impurities, they are not applicable to polar organic impurities present in small concentrations without preconcentration.

To determine toxic organic compounds (C_1–C_3 alcohols, acetone, methyl ethyl ketone and methyl isobutyl ketone), a method was developed previously for their headspace GC analysis with preliminary preconcentration in C_4–C_5 alcohols[1]. However, we experienced serious interference problems with impurities in the absorbent.

We have now developed a procedure combining headspace analysis with preconcentration in water and salting out from sodium chloride solution and applied it to atmospheric measurements of *n*-butanol, isobutanol, ethyl acetate and butyl acetate. The preconcentration of polar compounds in water is preferable for their analysis. In this instance the purity of the absorbent can easily be ensured and further preconcentration of an extracted impurity is possible by the salting-out procedure.

The solubilities of the compounds of interest in water are: *n*-butanol 9.0 (15°C), isobutanol 9.5 (18°C), ethyl acetate 7.66 (15°C) and butyl acetate 0.5% (w/w) (25°C)[2]. The enthalpies of evaporation of *n*-butanol and ethyl acetate are: 52.30 and 32.26 kJ mol^{-1}, respectively[2].

The distribution coefficient (K) of an organic compound between water and air is the ratio of its concentrations in water (C_w, mg l^{-1}) and air (C_a, mg m^{-3}), $K = C_w/C_a$. The volatility behaviour (F) of an absorbent obeys the condition[3] $FK < 0.5$, where $F = PM(RT\rho)^{-1}$, P = vapour pressure, M = molar mass, ρ = density of absorbent, R = gas constant and T = absolute temperature.

Here we report a method for the extraction of atmospheric impurities from water for the GC determination of polar compounds in an air matrix at environmental levels.

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EXPERIMENTAL

All reagents were of analytical-reagent grade. For the preconcentration procedure we used an absorption vessel fitted with a porous glass plate and containing 4 cm^3 of distilled water. A gas stream at a flow-rate of 400 cm^3 min^{-1} was introduced into the water through the porous plate.

The breakthrough volume of the gas to be analysed (air or standard mixture) is *ca.* 12 l. When equilibrium had been attained, a 1-cm^3 aliquot of solution was placed in a flask (*ca.* 15 cm^3) for gas extraction. Tubes served to extract the volatile organic compounds from the water samples into air. The gas extraction tube had previously been filled with 1 cm^3 of water (for butyl acetate) or a mixture of 1 cm^3 of water and 0.5 g of sodium chloride (for *n*- and isobutanol and ethyl acetate). Both tubes were sealed with rubber corks, fixed in metallic chucks and shaken for about 15 min. The resulting vapour phase (1 cm^3) was removed for GC analysis.

The measurements were carried with a Chrom-5 gas chromatograph equipped with a flame ionization detector and a glass column (1 m × 4 mm I.D.) of 15% polyethylene glycol 600 on Chezasorb AW. At flow-rates of the carrier gas (argon) of 25, hydrogen of 30 and air of 300 cm^3 min^{-1} and an oven temperature of 130°C, the retention times of *n*-butanol, isobutanol, ethyl acetate and butyl acetate were 7.0, 3.0, 1.5 and 4.0 min, respectively.

Standard gas mixtures were prepared by a stepwise procedure. A known volume of liquid alcohol or ester was placed in a calibrated capillary thermostat tube (1.15 mm I.D.). Nitrogen was used as a gas diluent, as the diffusion coefficients of organic compounds in air and nitrogen are similar. Stable concentrations of gaseous alcohols and esters may be generated at the 0.08–1.0 mg m^{-3} level by passing nitrogen over the surface in the capillary tube. The level of surface (and the amount of liquid evaporated) were determined to a precision of ±0.001 mm.

RESULTS AND DISCUSSION

A typical chromatogram of the vapour phase is given in Fig. 1. The calibration graphs are linear in the ranges 0.02–0.24 mg cm^{-3} for *n*- and isobutanol and butyl acetate and 0.01–0.3 mg cm^{-3} for ethyl acetate.

We established the distribution behaviour of the alcohols and esters in the nitrogen–water system and examined the temperature dependence (Table I). The distribution behaviour of organic compounds obeys the equation

$$\ln K = \triangle H^\circ/RT + \triangle S^\circ/R$$

where $\triangle H^\circ$ and $\triangle S^\circ$ are the standard enthalphy and entropy of extraction, respectively. The linear dependence between log K and $1/T$ allows the K values to be determined if the absorption temperature differs from the examined values (Table I). For comparison, the distribution coefficients in the nitrogen–*n*-butanol system are 1050, 5630 and 6770 for methanol, ethanol and *n*-propanol, respectively, and in the nitrogen–*n*-amylalcohol system they are 307, 580 and 1035 for acetone, methyl ethyl ketone and methyl isobutyl ketone, respectively.

The sampling temperature is limited by freezing of the absorbent in the porous

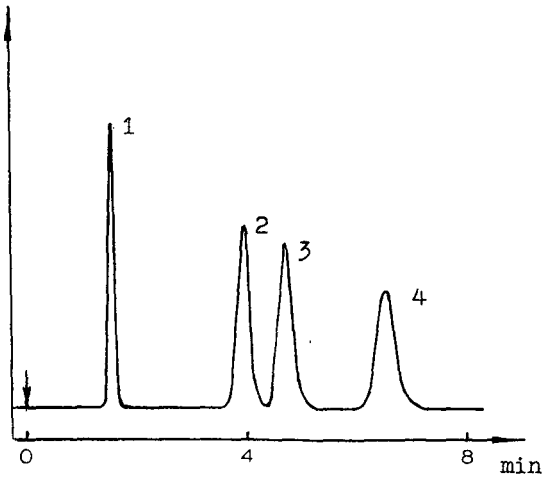

Fig. 1. Typical chromatogram of the vapour phase: 1 = ethyl acetate; 2 = isobutanol; 3 = butyl acetate; 4 = *n*-butanol.

TABLE I

DISTRIBUTION COEFFICIENTS IN THE NITROGEN–WATER SYSTEM

Temperature (°C)	*K*			
	n-Butanol	*Isobutanol*	*Ethyl acetate*	*Butyl acetate*
10	6250 ± 35	3238 ± 25	1125 ± 10	1660 ± 20
20	1220 ± 22	1038 ± 15	572 ± 8	1480 ± 11
25	897 ± 5	599 ± 6	450 ± 5	1080 ± 12
35	252 ± 10	205 ± 15	180 ± 7	814 ± 20
45	–	–	–	577 ± 18

plate and by the increase in gas solubility at low temperature. The range of measured concentrations depends on the absorption temperature (Table II).

The detection limits are 1.0, 5.0, 0.3 and 0.35 mg m^{-3} for methanol, ethanol, *n*- and isobutanol and ketones, respectively.

TABLE II

RANGE OF MEASURED CONCENTRATIONS OF ALCOHOLS AND ESTERS

Temperature (°C)	C_a (mg m^{-3})			
	n-Butanol	*Isobutanol*	*Ethyl acetate*	*Butyl acetate*
10	0.08–1.2	0.07– 1.1	0.08–1.2	0.08–1.2
20	0.41–6.1	0.23– 3.5	0.16–2.4	0.09–1.3
25	0.56–8.4	0.40– 6.0	0.20–3.0	0.12–1.8
35	2.0–30	1.2 –17.6	0.47–7.1	0.16–2.4

According to the concentration level expected, the sampling temperature can be determined. The detection limit depends on the absorption temperature. The analytical precision at 10°C is 7.0% for *n*- and isobutanol, 14.5% for ethyl acetate and 9.6% for butyl acetate.

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CHROM. 22 195

Note

Simple device for the determination of volatile chlorinated hydrocarbons in water by gas chromatography

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In trace analysis, methods for concentrating analyte compounds from the sample are often necessary. For this purpose, the dynamic headspace method is

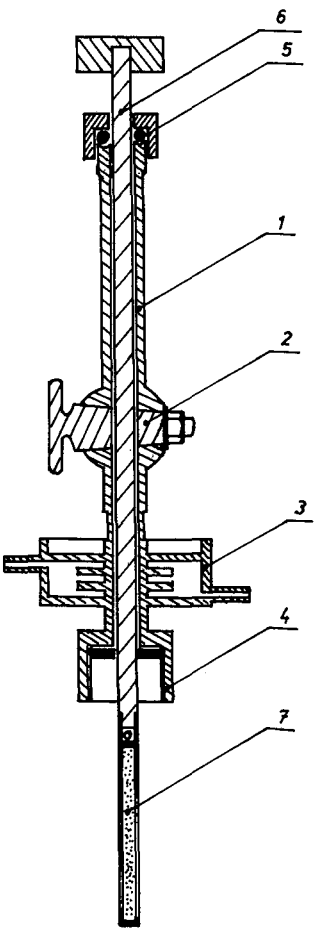

Fig. 1. Device for insertion of concentration microcartridge into the injector of the gas chromatograph.

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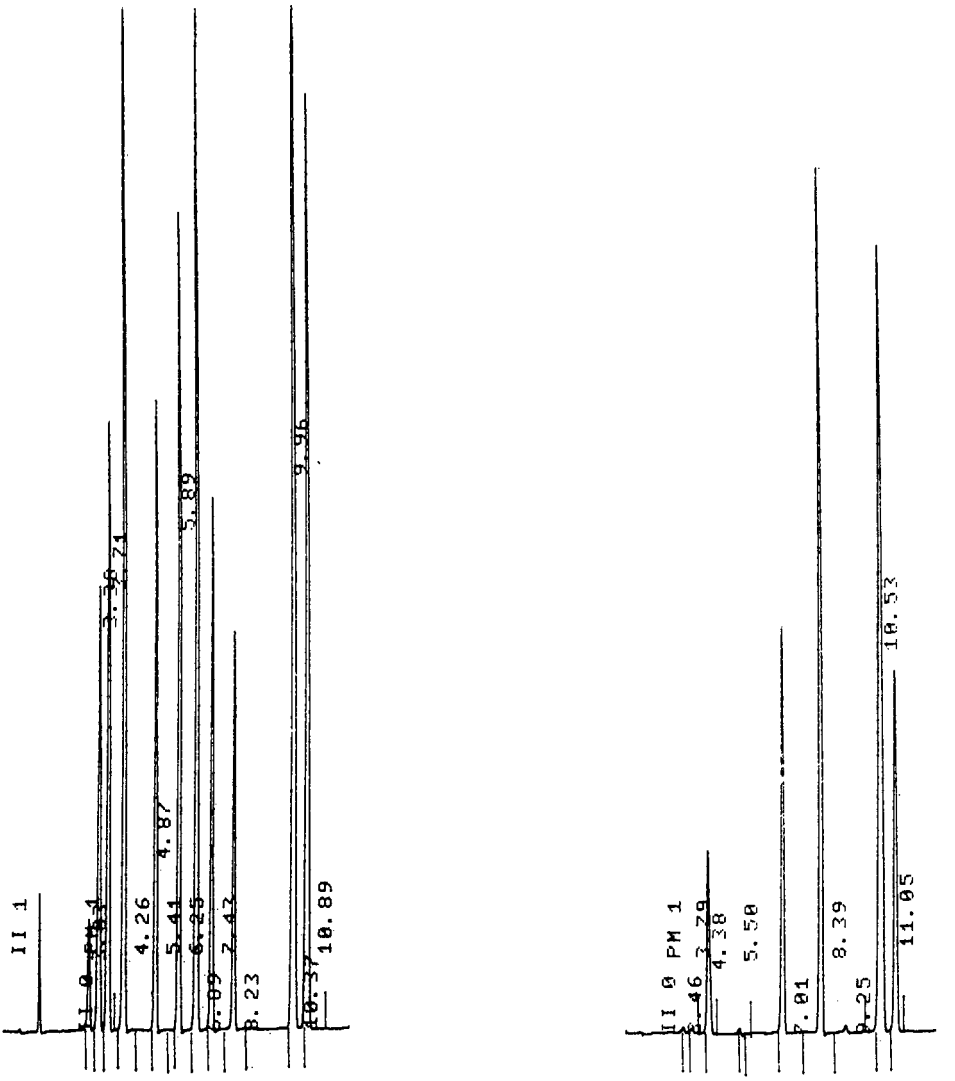

Fig. 2. Chromatogram of a calibration mixture for the determination of volatile aromatic and chlorinated hydrocarbons in water. Peaks: retention time 3.03 min = chloroform; 3.38 min = 1,2-dichloroethane; 3.71 min = benzene; 4.26 min = trichloroethylene; 4.87 min = toluene; 5.89 min = perchloroethylene; 6.89 min = chlorobenzene; 7.43 min = xylene; 8.23 min = *n*-nonane; 10.37 min = 1,4-dichlorobenzene; 10.89 min = 1,2-dichlorobenzene.

Fig. 3. Chromatogram of a real water sample. Peaks: retention time 3.46 min = 1,2-dichloroethane; 3.79 min = benzene; 4.38 min = trichloroethylene; 5.50 min = toluene; 7.01 min = chlorobenzene; 8.39 min = *n*-nonane; 10.53 min = 1,4-dichlorobenzene; 11.05 min = 1,2-dichlorobenzene.

suitable[1–3] but requires a special accessory, *i.e.*, a furnace with a controlling unit for thermal desorption of the analyte compounds.

We have designed a simple device (Fig. 1) for collecting the analyte compounds

in a preconcentration microcartridge, which can be inserted into the injector of any gas chromatograph with a sufficiently wide injector without interruption of the carrier gas flow[4].

The apparatus consists of a tube (1) with a tap (2), and is surrounded by a cooling rib (3) and a coil (4) for attachment to the injector of the gas chromatograph on one side, and a nut with a washer on the other side. A piston (6) with connected microcartridge (7) is inserted through this nut.

The chromatographic conditions adopted were as follows: gas chromatograph, Chrom 5 (Laboratory Instruments, Prague, Czechoslovakia); column, high-efficiency packed column (2.5 m × 3 mm I.D.) of 5% OV-101–Inerton Super (0.125–0.16 mm) (Lachema, Brno, Czechoslovakia); number of plates (80°C, isothermal run), 14 000 for octane and 19 000 for 1,4-dichlorobenzene; column temperature, 40°C for 2 min, then increased to 65°C, and finally at 7.5°C/min to 190°C; carrier gas, nitrogen at 19 ml/min; flame ionization detector, electrometer attenuation 1/128; integrator, Spectra-Physics 4200; and chart speed, 0.5 cm/min.

This method was applied to the determination of volatile chlorinated hydrocarbons in water. The analyte compounds were isolated from a 50-ml water sample by the dynamic headspace method by concentration on the micocartridge and inserted into the injector of the gas chromatograph.

The minimum concentration of chloroform, 1,2-dichloroethane, perchloroethylene and trichloroethylene that can be determined is 0.2 $\mu g\ l^{-1}$, of chlorobenzene, 1,2- and 1,4-dichlorobenzene 0.1 $\mu g\ l^{-1}$ and of benzene and toluene 0.05 $\mu g\ l^{-1}$.

The utilization of flame ionization detection (FID) for routine water quality control is advantageous with regard to the possibility of the simultaneous determination of halogenated hydroarbons and other volatile compounds. Compared with electron-capture detector, FID is not as sensitive to different interferences (*e.g.*, carrier gas purity, effects of water vapour). The determination of volatile hydrocarbons and chlorinated hydrocarbons is illustrated by the separation of a calibration mixture in Fig. 2 and of a river-water sample in Fig. 3.

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CHROM. 22 160

Application of gas chromatography with electron-capture detection to trace analysis of halogenated compounds

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ABSTRACT

The construction, operation and examples of applications of the automatic AF-1 analyser for the trace analysis of halogenated compounds in air and other gases are described. The instrument is a gas chromatograph containing the electron-capture detector with a sensitivity of 10^{-6} ppm for strongly electronegative compounds, such as SF_6 and Freons. The analyser is useful for trace investigations when halogenated compounds are applied as tracers in the determination of ventilation rates and dispersion patterns within coal mines etc. and in the tightness investigations of industrial installations.

INTRODUCTION

A schematic diagram of a tracer experiment is shown in Fig. 1. The data obtained allow one to establish a relationship between the "input" and "output" of an investigated object and permit the calculation of a mathematical model or several physical parameters, such as the flow velocity of gaseous streams, air exchange rate in at low air flows, transit air time, recirculations, mean residence time of the tracer and dispersion rate, depending on the kind of objects investigated.

Numerous organic and inorganic materials have been used as tracers but most

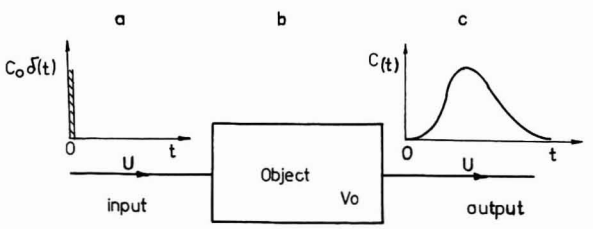

Fig. 1. Schematic diagram of tracer experiment. (a) Tracer δ-Dirac's injection form on the investigated object input; (b) investigated object, V_0 = volume, u = rate of air flow; (c) the tracer residence time distribution function at the investigated object output.

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have certain inherent drawbacks. At low concentrations chemical tracers are not detected as easily and they are often highly adsorbed on many surfaces. Radioactive substances can be detected at low concentrations, but they are difficult to handle and likely to be unacceptable to persons working near the objects being investigated.

A useful tracer gas must be detectable at low concentrations, must be safe, odourless, chemically and thermally stable, and should not occur naturally in the environment. Sulphur hexafluoride (SF_6) and some Freons, such us F12B1, F12B2 and F114B2, meet these essential requirements. Halogenated compounds, with electron-capture constants k_1 around 10^{-7} cm^3/s, can be detected at a very low concentrations ($<10^{-6}$ ppm) by gas chromatography with electron-capture detection. Halogenated tracer techniques have been shown to be useful in air and gas migration studies in meteorology and aerology[1–4], and also in oceanography[5,6] and hydrology[7,8].

Some of the Freons, such as F12, F11, F113 and F10, now occur "naturally" in the environment[9]. The application of these Freons as tracers is possible at a concentration about 100 times higher than that of the other freons, to eliminate the influence of their background concentration.

EXPERIMENTAL

The AF-1 analyser is a portable gas chromatograph equipped with an electron-capture detector, operated at constant frequency, and a strip-chart recorder. Fig. 2 shows the instrument and a its circuit diagram is presented in Fig. 3. The mea-

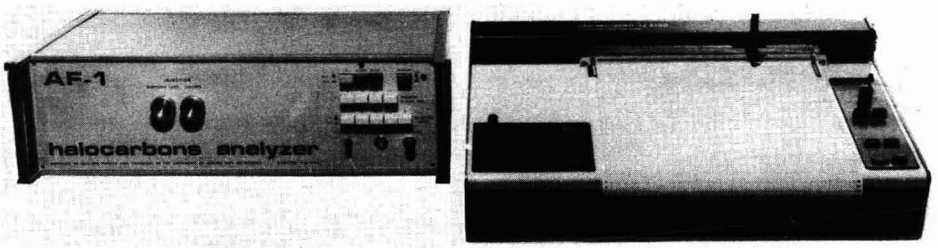

Fig. 2. Photograph of the AF-1 halocarbons analyser. Weight, 15 kg. Dimensions, 350 × 200 × 400 mm.

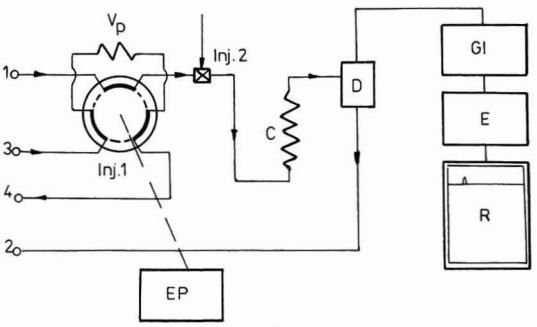

Fig. 3. Circuit diagram of the AF-1 analyser.

TABLE I

OPERATING CONDITIONS

Tracer	*Detector temperature (K)*	*Column temperature (K)*	*Column length (m)*	*Column packing*	*Carrier gas*
SF_6	300	300	1	Silica gel or molecular sieve 5 A	N_2 or Ar + 10% CH_4, 40 ml/min
Freons F12 or F12B1, F11 or F12B2, F113, F114B2, F10	573	300	3	10% DC 200 on chromosorb W	N_2 or Ar + 10% CH_4, 60 ml/min

surement unit contains two gas circulations: the carrier gas path (1 and 2) and the investigated gas path (3 and 4). The carries gas path includes the automatic gas sample injector Inj 1, the manual syringe injector Inj 2, the gas chromatographic column C and the electron-capture detector D. The investigated gas path includes the automatic gas sample injector Inj 1 and sample loop V_p. The measuring unit also contains all associated electronics, *i.e.*, the detector supply pulse generator GI, elec-

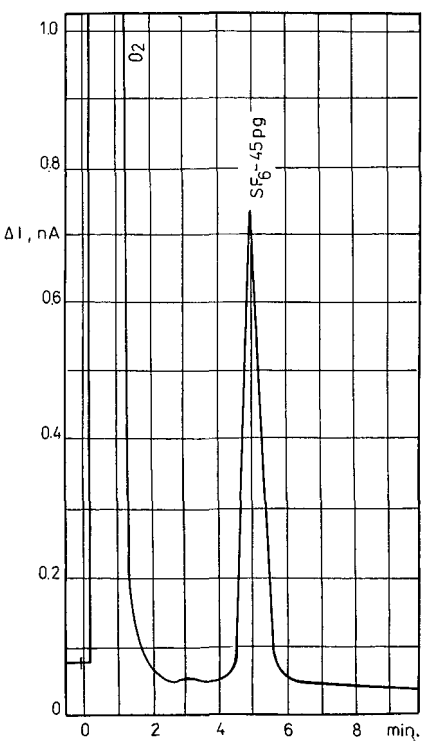

Fig. 4. Example of SF_6 analysis in a 5-ml air sample.

TABLE II

TECHNICAL PARAMETERS OF THE AFI ANALYSER WITH SF_6 AS TRACER

Parameter	*Value*
Gas sample injection volume	5 ml
Detection limit for SF_6	10^{-13} g/ml
Carrier gas and flow-rate	High-purity N_2 or Ar + 10% CH_4 (O_2 < 1 ppm), 40 ml/min
Detector β-source	^{63}Ni, 15 mCi
Linear range	100
Chromatographic column	1 m, silica gel
Detector and column temperature	300 K
Sampling and injection	Manually or automatically every 15 min

trometer E and time programmer EP that controls he automatic sampling operation system Inj 1. The strip-chart recorder R serves for monitoring the detector output. The operating conditions are given in Table I.

RESULTS

An example of the analysis of an air sample with SF_6 as the tracer is presented in Fig. 4. and technical parameters are given in Table II. The analysis of the back-

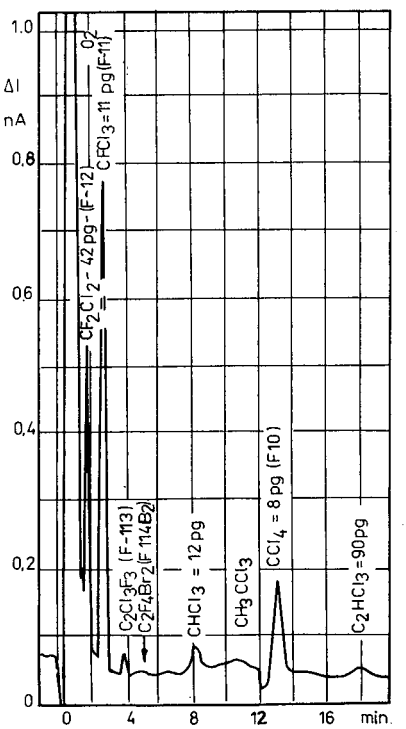

Fig. 5. Example of background analysis of halogenated compounds in a 5-ml air sample.

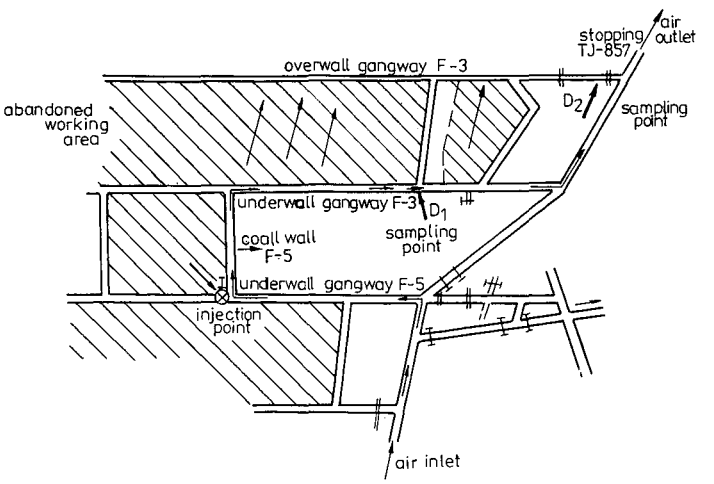

Fig. 6. Map of excavation mine: I = tracer injection point; D_1, D_2 = sampling points.

ground concentration of some freons in air is shown in Fig. 5. A very low detectability range at the level 10^{-12} g/ml for strongly electronegative compound makes possible the direct analysis of gaseous samples without enrichment.

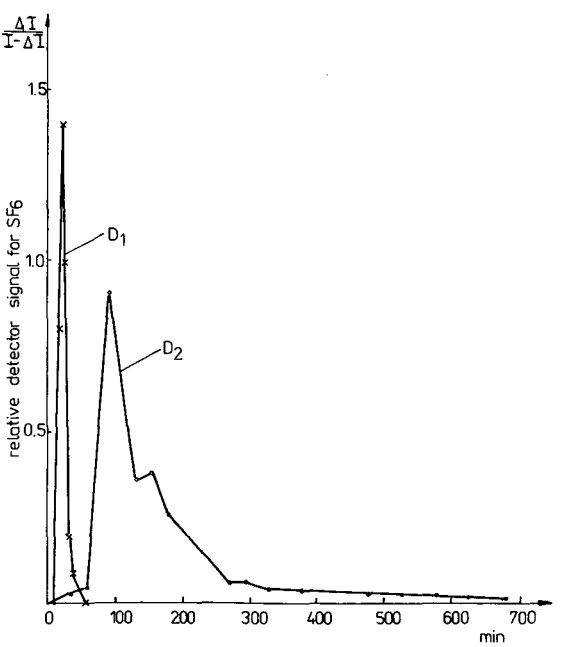

Fig. 7. Tracer concentrations at points D_1 and D_2 (Fig. 6) as a function of time from the moment of injection.

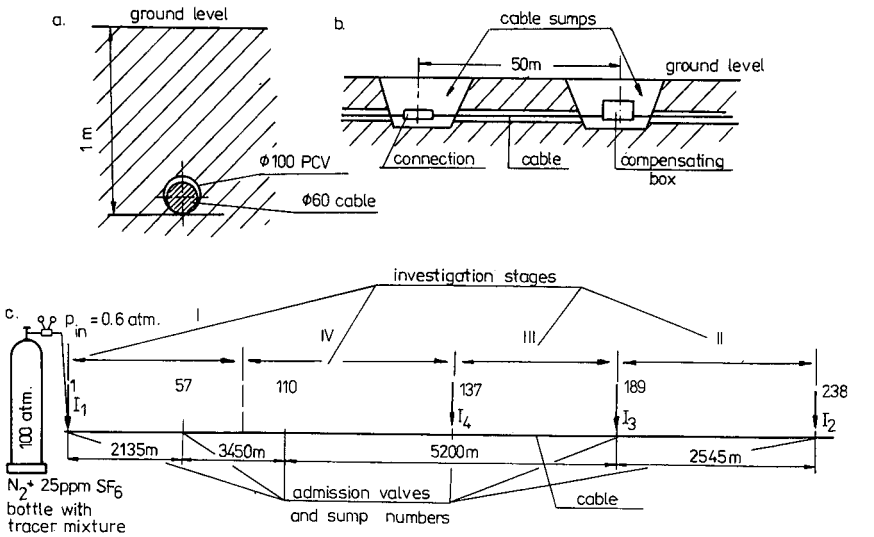

Fig. 8. Tightness investigations of telecommunication cable coating (a), (b) location of investigated cable; (c) the thightness investigation stages.

Application to the investigation of the migration of air in mines (ref. 10)

The results of air and gas flow investigations in a coal mine are shown in Figs. 6 and 7. The tracer method was used to demonstrate that there is air migration through abandoned workings wall F-3 in the direction of isolation stopping TJ-857. In each injection, 1 l of SF_6 was introduced into the flowing air at point I. The air at points D_1 and D_2 was sampled using glass syringes and analysed with the AF-1 analyser. Fig. 7 illustrates the changes in the tracer concentration at points D_1 and D_2 as a function of time, starting from the moment of injection. The following results were obtained.

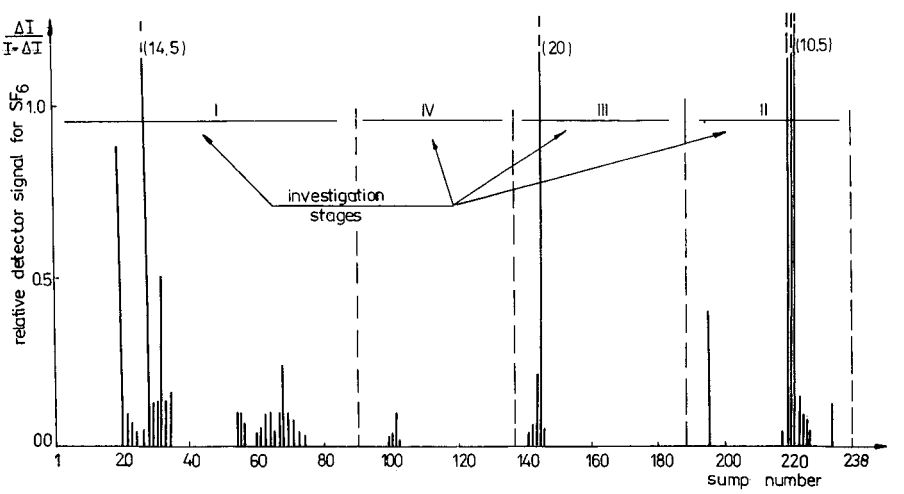

Fig. 9. Tracer concentrations in each cable sump as the result of tightness investigations.

The transit time for the tracer from the injection point I to point D_1 is 20 min, hence the average air flow velocity for this distance of 920 m is 0.75 m/s. In the abandoned workings wall F-3 there are two main gas migration passages, which is evident from two maxima excluding the main maximum. The abandoned workings wall F-3 is leaky, *i.e.*, in addition to two passages there are other connections, as shown by the "tail" of tracer residence time distribution curve.

Application to tightness localization of telecommunication cable coating

The set-up is shown in Fig. 8a and b. The cable of length about 14 km I.D. 60 mm is located 1 m underground in a PVC pipe of I.D. 100 mm and passes through 238 cable sumps. The cable contains 86 connections and eight compensation boxes which balance its wave resistance. Because of the length of the cable, its tightness investigations were divided into four stages (see Fig. 8c). In each stage a tracer mixture of nitrogen containing 25 ppm of SF_6 was injected continuously into the cable from a high-pressure bottle via admission valves I. The air from each cable sump was then sampled with 50-ml glass syringes and analysed using the AF1 analyser.

The results of these qualitative investigations, *i.e.*, the dependence of the relative detector signal, $\Delta I/(I - \Delta I)$ (where I is the detector standing current) on the cable sump number for each investigation stage are illustrated in Fig. 9. Three large leaks were found from the compensation boxes (sumps 27, 144 and 220) and several smaller leaks from the cable connections.

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Comparison of sorbents for solid-phase extraction of polar compounds from water

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ABSTRACT

Octadecyl-bonded silica, styrene–divinylbenzene and hydroxyethyl methacrylate–ethylene dimethacrylate sorbents were tested for their ability to preconcentrate aniline, benzothiazole, cyclohexanone and cyclohexanol. Breakthrough curves for these compounds were measured for aqueous solutions at pH 5.8 and 10.0 by gas chromatographic analysis of fractions of the aqueous effluents. Elution curves of the analytes in methanolic eluates were obtained in order to determine the optimum volume of the eluate. Using breakthrough volumes and widths of the elution curves, theoretical preconcentration factors were calculated for all analyte–aqueous sample–sorbent systems tested. Octadecyl-bonded silica and styrene–divinylbenzene sorbents were found to be suitable for the preconcentration of weakly polar to polar compounds for practical water quality control purposes.

INTRODUCTION

Sample preparation is an important step in the analysis of complex water matrices for organic pollutants. Many techniques are available for the isolation and preconcentration of these compounds, and solid-phase extraction is currently being intensively developed. Many examples of the use of solid sorbents for the accumulation of organics from aqueous solutions can be found in the literature and have been well reviewed[1–5].

With regard to general methodology, solid-phase extraction (SPE) procedures can be performed in two ways: off-line using disposable cartridges and on-line via pre-column switching. Although automation of the sorbent extraction techniques,

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incorporating them into multi-dimensional chromatography/column switching systems, offers a very effective tool for analyses of water samples, for some applications of trace enrichment disposable cartridges still have significant advantages owing to their ease of handling and portability. The easy manipulation of these cartridges permits processing of the samples in the field, which avoids the transport and possible breakage of samples in glass containers. Moreover, the sorbed organic compounds are well preserved against breakdown by bacteria[6].

The solid supports mostly used in the SPE are bonded silicas and various polymeric resins. Their application includes the preconcentration of polycyclic aromatic hydrocarbons[7–10], pesticides[11–18], chlorophenols[19–21], phenols[9,21], polychlorinated biphenyls[13], priority pollutants[22], organosulphur compounds[23], nitroaromatics[24], surfactants[25,26], chloroanilines[27] and nitrogen heterocycles[9].

The polarity of accumulated compounds usually ranges from weakly polar to non-polar. Efforts to capture more polar compounds by means of hydrophobic sorbents often lead to a drastic decrease in recovery, so that for binding of these types of organic compounds other types of interactions (*e.g.*, ionic) have to be used.

The factor that most influences the accumulation of the analyte in the SPE column is its retention, which can be expressed as the capacity factor of the analyte in the water–stationary phase (sorbent) system. It has been well documented that even for columns that have a small number of theoretical plates, which is typical in SPE, high breakthrough volumes can be obtained if the retention is sufficiently high[28].

In order to obtain an effective preconcentration procedure, maximization of the amount of water sample and minimization of that of the eluting solvent are required. An approach to the development and verification of an SPE method has been reported[17]. Another possible approach is to measure the breakthrough curve for the analyte–water–sorbent system and concentration profile of the analyte in the organic eluent–sorbent system (*i.e.*, the retention characteristics of the sorbent bed in the presence of two extreme mobile phases). Having obtained information on these retention characteristics, suitable volumes of the water sample and the organic eluent can be determined.

The objective of this work was to evaluate, using the latter approach, two popular types of solid supports, octadecyl-bonded silica and styrene–divinylbenzene copolymer, for their capacity for selected polar compounds. Another solid polymer support, Spheron 100, a copolymer of hydroxyethyl methacrylate and ethylene dimethacrylate, which is more polar than styrene–divinylbenzene resin was chosen for testing as it was assumed that it would have higher affinity for polar molecules. In this instance, however, the presence of water as the competitive factor might play a negative role and this had to be taken into account.

EXPERIMENTAL

Materials

Model solutions of cyclohexanol, cyclohexanone, aniline and benzothiazole (Lachema, Czechoslovakia) in water were prepared from stock solutions in methanol (5000 ppm). These stock solutions were also used to spike industrial wastewaters with the pollutants for overall recovery tests and the same stock solutions were diluted with methanol to serve as a reference in all recovery tests. Deionized water was

obtained from a Rodem purifier system (OPP Tišnov, Czechoslovakia). Potassium chloride, potassium carbonate and dimethylaniline (Lachema), used as an internal standard, were of analytical-reagent grade.

Silica-Cart C_{18} cartridges (60 μm) and empty polypropylene cartridges were obtained from Tessek (Prague, Czechoslovakia). Synachrom E 5 styrene–divinylbenzene resin was obtained from Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences (Prague, Czechoslovakia) and was ground and sieved to obtain a few grams of 40–71 μm particle size fraction. Spheron 100 hydroxyethyl methacrylate–ethylene dimethacrylate copolymer resin (25–40 μm) was purchased from Lachema. Polymer resins were wet-packed into polypropylene cartridges.

Apparatus

An FCC 60 fraction collector (Laboratory Instruments Works, Czechoslovakia) and an MMC-1 pump (Microtechna, Czechoslovakia) were used for collection of water fractions to measure breakthrough curves. Concentrations of analytes in water fractions were measured using a CHROM-4 gas chromatograph (Laboratory Instruments Works) equipped with a flame ionization detector. Integration was done on a CI-100 integrator (Laboratory Instruments Works). The chromatographic conditions were as follows: glass column, 2 m × 3 mm I.D., packed with 10% Carbowax 20M on Inerton N-AW (0.125–0.160 mm) (Lachema); carrier gas, nitrogen at 30 ml/min; injector temperature, 160°C; column temperature, dependent on the compound; and volume injected (direct injection of water sample), 2 μl.

Desorptions were carried out using an M122 Doser pump (Mikrotechna) with glass syringe as a solvent reservoir. Analyses of methanolic eluates were done on a Hewlett-Packard (Waldbronn, F.R.G.) 5880 Level Four gas chromatograph equipped with a flame ionization detector and a split/splitless injector. The chromatographic conditions were as follows: 20 m × 0.25 mm I.D. Carbowax 20M glass capillary column (Lachema); carrier gas, nitrogen at 3 ml/min; temperature programme, 10 min at 90°C, then increased at 6°C/min to 190°C; injector temperature, 210°C; and detector temperature, 250°C.

Procedure

The disposable cartridge with a volume of 1 ml containing C_{18}-silica, Synachrom E 5 or Spheron 100 sorbent was activated by rinsing with 15 ml of methanol, which was subsequently displaced with 3 ml of water. The conditioned cartridge was then used for breakthrough, desorption or overall recovery experiments. To measure a breakthrough curve, a 5 ppm aqueous solution of the analytes was pumped through the column at a flow-rate of 5 ml/min and the effluent was collected in glass vials. At the beginning, the volume of each water fraction collected was 5 ml. After 150 ml of effluent had passed through the column, the volume of the fractions collected was increased to 15 ml. A 2-μl aliquot of each fraction was analysed by gas chromatography (GC).

The breakthrough characteristics were measured for a solution in deionized water (pH 5.8) and for an aqueous solution with the pH adjusted to 10.0 with potassium carbonate. To permit the GC determination of cyclohexanone in an alkaline water fraction, this fraction had to be neutralized with a few microlitres of hydrochloric acid. To obtain the shape of a desorption curve, a cartridge with accumulated

analytes was used. For this purpose 50 ml of a 5 ppm aqueous solution of all four compounds were passed through an activated disposable column. The residual water was removed by drying the cartridge under vacuum for 25 min. The cartridge was then connected in the backward-flush mode to the luer of the glass syringe in the pump and to the outlet of this cartridge a thin needle with a cut end was attached. The methanolic effluent was collected in vial as ten-drop fractions. An average volume of ten drops of the methanolic fraction of *ca.* 60 μl was calculated from the average weight of ten drops and the density of methanol. Aliquots of 2 μl of the effluent fractions were analysed by GC. Quantitative analyses in the breakthrough and desorption experiments were performed via the calibration graph method.

For overall recovery tests, 30 ml (60 ml for Synachrom E 5 cartridges) of a 200 ppb[a] solution of the analytes in deionized water was forced through the activated cartridge with suction at a flow-rate of 5 ml/min. The sorbed compounds were eluted with methanol. For desorption wet cartridges were used in order to prevent possible losses of cyclohexanone and cyclohexanol caused by drying, which had been observed in our previous work[29]. The volume of the aqueous fraction of the eluate to be discarded was determined from the weight difference between wet and dry cartridges and was controlled during the elution. The second, methanolic, fraction (600 μl for C_{18} and Spheron 100 and 1200 μl for Synachrom E 5) was collected separately and analysed.

Wastewater samples were adjusted to pH 10.0 with potassium carbonate and the necessary volume of stock solution was added to increase the analyte concentrations to at least 200 ppb. The subsequent sample processing was similar to that for model water samples. In overall recovery tests prior to the analysis, N,N-dimethylaniline was added to the methanolic eluate as an internal standard.

RESULTS AND DISCUSSION

Cyclohexanone, cyclohexanol, aniline and benzothiazole can be found in industrial wastewaters and can subsequently pollute surface waters. Their detection and quantification are important, *e.g.*, for testing water quality and for evaluation of wastewater treatment plant efficiency. Their isolation from aqueous solution using a hydrophobic medium (solvent or sorbent) can be problematic owing to their relatively high solubility in water (several grams per 100 g of water). Moreover, there is a possibility of ionization of aniline (pK_b = 9.4) and benzothiazole (pK_b = 12.4), which leads to a dependence of the recovery on the sample pH. These potential problems mean that the application of SPE in such instances has to be well verified so as not to obtain a method with poor reproducibility and low efficiency.

It is clear that the overall SPE recovery of an analyte is a function of both retention efficiency and elution efficiency. The most laborious but most exact approach to maximizing the overall efficiency is to measure the breakthrough curve and the analyte concentration profile in the eluate (elution curve). From the breakthrough curve the breakthrough volume can be calculated, which corresponds to the maximum volume of the water sample that can be forced through the column without losses of analytes. From the elution curve the minimum amount of eluent needed to

[a] Throughout this article, the American billion (10^9) is meant.

remove the sorbed analyte quantitatively can be determined. Thus, for any SPE application, the maximum theoretical preconcentration factor can be found, given by $F = V_B/W$, where V_B (ml) is the breakthrough volume and W (ml) is the width of the elution curve.

Examples of the calculation of the breakthrough volume can be found in the literature[28,30]. To obtain the width of an elution curve for calculating F, a volume interval of the eluate can be chosen that covers 99% (or 95%) of the total area below the elution curve, *i.e.*, a volume that contains 99% (or 95%) of the desorbed solute mass. In practice, however, where preconcentration of several compounds with different positions of the elution curve on the volume axis is the most common situation, the W value applied should include a volume interval containing all elution curves, otherwise losses of some compounds will occur. This concept, naturally, leads to a decrease in the actual preconcentration factor. Nevertheless, the proposed theoretical W value is useful for characterizing the sorbent as the average F value for any sorbent can serve for the approximate evaluation of its preconcentration efficiency towards a certain group of analytes (*e.g.*, polynuclear aromatic hydrocarbons, priority pollutants, polar compounds).

The breakthrough curves of the investigated polar compounds on C_{18}-silica, Synachrom E 5 and Spheron 100 are shown in Figs. 1–3, which were obtained from experiments with (a) non-buffered deionized water of average pH 5.8 and (b) buffered deionized water adjusted to pH 10.0. The pH of the pure deionized water samples was measured twice during a breakthrough experiment, first before the water sample had been forced through the column and then immediately after the sorption was finished. All the pH values were found to be constant within the range 5.7–5.9. The non-adjusted deionized water used in the first part of the experiments was chosen to exclude the influence of any possible interferences (*e.g.*, ions including those of buffer solutions). Moreover, the average pH of this non-adjusted water could be considered to be approximately the lowest end of the interval of pH values commonly occurring in real surface waters. The pH of 10.0 used in the second part of the breakthrough experiments to suppress the ionization included the upper end of that real pH interval, which is usually 6–8.

From the displayed breakthrough curves the maximum applicable volumes of the water sample can be calculated. The exact volume depends on what breakthrough level (the ratio of the outlet to the inlet concentration) is taken as the operational value.

The breakthrough curves shown in Figs. 1–3 represent mean values from three measurements. The relative standard deviation is 5–10% for each sorbent–analyte–sample pH system.

According to our assumptions, alkalinization of a water sample should have resulted in an increase in the breakthrough volume for weakly basic benzothiazole and aniline owing to suppression of the ionization. The results obtained, however, did not confirm our expectations and the increase in retention did not occur. Moreover, particularly for benzothiazole, a considerable decrease in its retention on C_{18} and Synachrom E 5 with increase in pH was observed. On the other hand, alkalinization of the water sample led to a drastic increase in the breakthrough volume of cyclohexanone and the breakthrough curve of cyclohexanone in alkaline solutions was less steep than all the other curves. This behaviour might be a consequence of a keto–enol

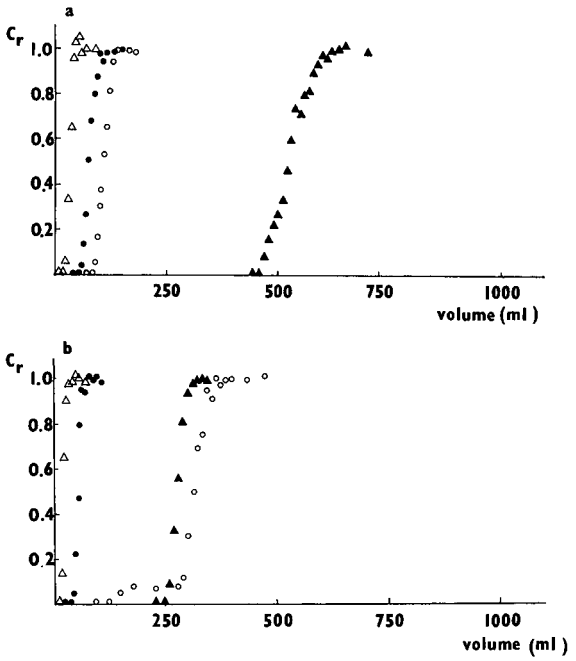

Fig. 1. Breakthrough curves of cyclohexanone (○), cyclohexanol (●), aniline (△) and benzothiazole (▲) on Silica-Cart C_{18} cartridge, measured as relative concentrations of these compounds in aqueous effluent fractions. $C_r = C_e/C_i$, where C_e and C_i are the concentrations of the analytes in the effluent and influent, respectively. pH of the water: (a) 5.8 and (b) 10.0.

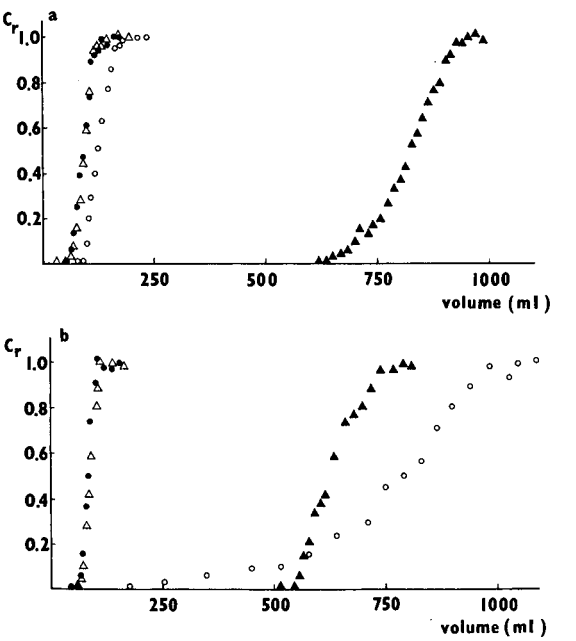

Fig. 2. Breakthrough curves of cyclohexanone (○), cyclohexanol (●), aniline (△) and benzothiazole (▲) on Synachrom E 5 cartridge. Details as in Fig. 1.

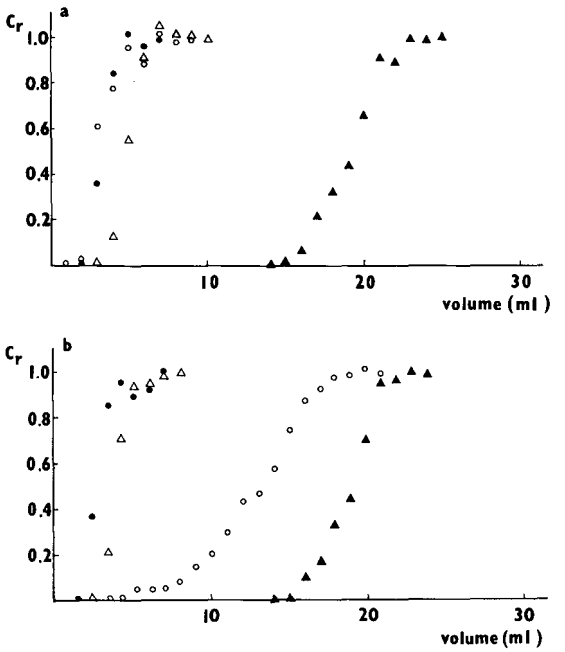

Fig. 3. Breakthrough curves of cyclohexanone (○), cyclohexanol (●), aniline (△) and benzothiazole (▲) on Spheron 100 cartridge. Details as in Fig. 1.

tautomerism of cyclohexanone. The enol-form, cyclohex-1-en-1-ol, should have a much higher affinity towards hydrophobic surfaces. The practical consequence of this phenomenon is much increased capacity for cyclohexanone.

From the comparison of investigated sorbents it is obvious that whereas Synachrom E 5 and C_{18} have similar retention properties, Spheron 100 is much less efficient. A possible reason for this behaviour is water acting as a competing agent during the sorption.

The elution curves of the investigated compounds (Figs. 4–7) begin on the volume axes in the first or second methanolic fraction, except for the curve for cyclohexanone on C_{18}-silica. (Fig. 4a). From the curves obtained it can be seen that the width of an elution curve could in some instances be considered to be proportional to the retention ability of a given sorbent determined from breakthrough experiments. The differences between these elution curve widths, however, are not as great as those between corresponding breakthrough volumes. On the other hand, this proportionality is not valid for the elution curves of cyclohexanone on Synachrom E 5 and aniline on C_{18}, where the relative retention in the methanol–sorbent system is higher than that in the water–sorbent system in comparison with other cases. The considerable tailing of aniline on C_{18} might be ascribed to a secondary retention mechanism between residual silanol groups on chemically bonded silica and amines.

Theoretical preconcentration factors for the studied organic compounds were calculated according to the above equation $F = V_B/W$. Breakthrough volumes were calculated for a breakthrough level of 10%. Widths of the elution curves were obtained as the interval containing 99% of the area below the curve. Calculated F values

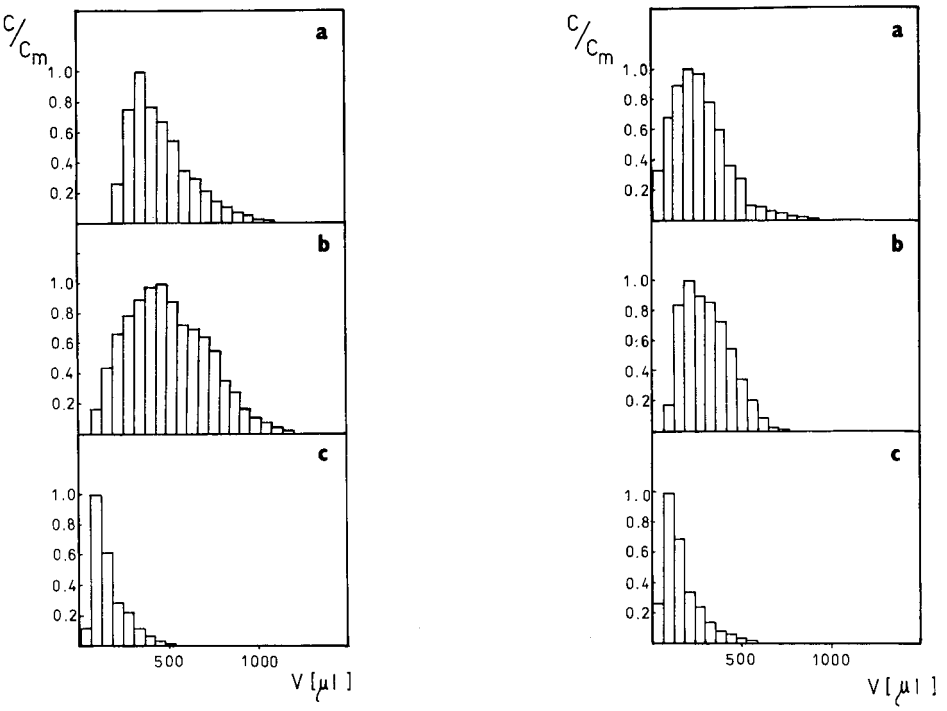

Fig. 4. Elution curves of cyclohexanone on (a) C_{18}, (b) Synachrom E 5 and (c) Spheron 100, measured as its concentration in methanolic effluent fractions. C and C_m are the concentration of cyclohexanone in a particular fraction and the maximum concentration of cyclohexanone in the particular fraction, respectively.

Fig. 5. Elution curves of cyclohexanol. Details as for cyclohexanone in Fig. 4.

for non-alkalinized and alkalinized samples and the mean values for each sorbent are listed in Tables I and II. These mean preconcentration factors reflect the general ability of the sorbent used to preconcentrate a given group of compounds. For our small group of compounds they serve only as an illustrative example, but they can help in establishing the general suitability of a chosen sorbent for the preconcentration of a large group of analytes.

To confirm the real preconcentration capabilities of the tested sorbents, the overall recoveries of the studied polar compounds from both non-alkalinized and alkalinized water samples were measured. As an additional test, the influence of the salting-out effect on the overall recovery was studied, 40 g/l of potassium chloride being added to an alkalinized sample. Table III shows the results of these experiments which confirmed C_{18} and Synachrom E 5 to be suitable for the preconcentration of the studied compounds. Except for aniline, the recoveries on C_{18} are similar for alkalinized and non-alkalinized samples, the differences being within the standard deviation range. Adjusting the pH to 10.0 did not result in any substantial change in the overall recoveries on Synachrom E 5. The low recoveries observed with Spheron 100 were caused by lower breakthrough volumes in comparison with the volume of

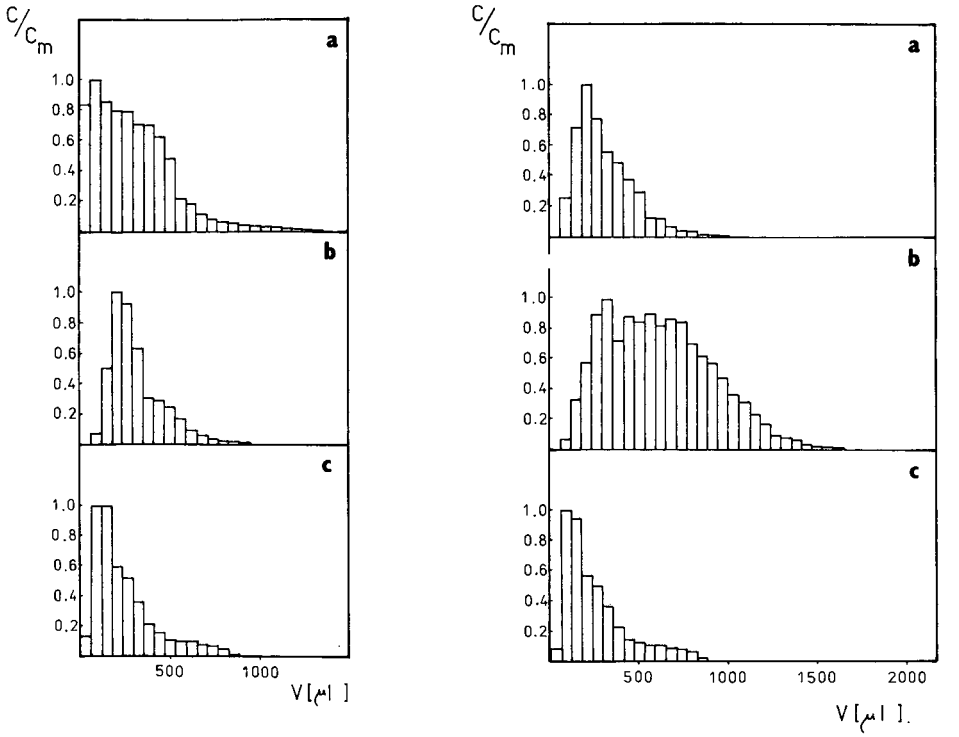

Fig. 6. Elution curves of aniline. Details as for cyclohexanone in Fig. 4.

Fig. 7 Elution curves of benzothiazole. Details as for cyclohexanone in Fig. 4.

water applied. For the recovery of aniline on C_{18}, it is interesting that the alkalinization of the water sample led to an increase in the recovery of aniline and, moreover, to a large increase in the reproducibility of the recovery, even though the breakthrough volumes in both instances were similar (for non-alkalinized water samples the average reproducibility of the recovery of aniline was 8–9 times worse than those observed for the other compounds, which were usually 3–5%). This behaviour could be ascribed to the different reactivities of free silanol groups under these conditions, which might be

TABLE I

THEORETICAL PRECONCENTRATION FACTORS OF CYCLOHEXANONE, CYCLOHEXANOL, ANILINE AND BENZOTHIAZOLE ON DIFFERENT SORBENTS AT pH = 5.8.

Valves calculated according to the equation $F = V_B/W$.

Compound	C_{18}	*Synachrom E 5*	*Spheron 100*
Cyclohexanone	150	115	4
Cyclohexanol	100	140	4.5
Aniline	32	130	4.5
Benzothiazole	680	600	20
Mean	240.5	246.3	8.3

TABLE II

THEORETICAL PRECONCENTRATION FACTORS OF CYCLOHEXANONE, CYCLOHEXANOL, ANILINE AND BENZOTHIAZOLE ON DIFFERENT SORBENTS AT pH = 10.0.

Values calculated according to the equation $F = V_B/W$.

Compound	C_{18}	*Synachrom E 5*	*Spheron 100*
Cyclohexanone	365	740	15
Cyclohexanol	60	120	4
Aniline	19	130	3.5
Benzothiazole	335	450	19
Mean	194.8	360	10.4

suppressed by increasing the pH. As can be seen in Table III, an increase in the ionic strength by means of potassium chloride caused no substantial changes in SPE recoveries.

Both C_{18} and Synachrom E 5 sorbents were used to preconcentrate benzothiazole, aniline, cyclohexanone and cyclohexanol from spiked treated wastewaters. The pH of all samples was adjusted to 10.0 with potassium carbonate to secure suitable conditions for accumulation of aniline. The results of these experiments (Table IV) confirmed that C_{18} and Synachrom E 5 are suitable preconcentrating media for studied polar compounds.

TABLE III

RECOVERIES OF CYCLOHEXANONE, CYCLOHEXANOL, ANILINE AND BENZOTHIAZOLE FROM FORTIFIED WATER ON THE INVESTIGATED SORBENTS

The volume of the water samples was 60 ml for Synachrom E 5 and 30 ml for C_{18} and Spheron 100.

Sample	*Compound*	*Recovery (%)*		
		C_{18}	*Synachrom E 5*	*Spheron 100*
A[a]	Cyclohexanone	88	88	10
	Cyclohexanol	89	92	10
	Aniline	64	78	15
	Benzothiazole	84	75	60
B[b]	Cyclohexanone	92	93	30
	Cyclohexanol	93	90	10
	Aniline	92	90	15
	Benzothiazole	82	72	75
C[c]	Cyclohexanone	90	90	35
	Cyclohexanol	89	89	12
	Aniline	90	92	20
	Benzothiazole	81	70	75

[a] Deionized water at pH 5.8.
[b] Deionized water adjusted to pH 10.0 with K_2CO_3.
[c] Deionized water adjusted to pH 10.0 with K_2CO_3, KCl added at a concentration of 40 g/l.

TABLE IV

RECOVERIES OF CYCLOHEXANONE, CYCLOHEXANOL, ANILINE AND BENZOTHIAZOLE FROM SPIKED WASTEWATERS ON C_{18}-SILICA AND SYNACHROM E 5

Wastewaters were adjusted to pH 10.0 with K_2CO_3. Volumes applied as in Table III.

Compound	*Recovery (%)*	
	C_{18}	*Synachrom E 5*
Cyclohexanone	92	89
Cyclohexanol	88	91
Aniline	93	88
Benzothiazole	75	77

CONCLUSION

Of the sorbents tested, Synachrom E 5 and octadecyl-bonded silica showed adequate preconcentration capabilities for benzothiazole, aniline, cyclohexanol and cyclohexanone. Although the preconcentration factors obtained are not as large as those which can be acheived on these sorbents with non-polar compounds, they are sufficient for practical purposes of water quality control. The method for calculating theoretical preconcentration factors from measured breakthrough and elution curves gives better information on the preconcentration capabilities of a given sorbent and allows optimum conditions for SPE procedures to be chosen.

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Use of polymeric sorbents for the off-line preconcentration of priority pollutant phenols from water for high-performance liquid chromatographic analysis

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ABSTRACT

The use of porous polymeric minicolumns for the determination of phenols from the U.S. Environmental Priority Pollutant List was studied. For the off-line preconcentration of priority pollutant phenols from water by solid-phase extraction, minicolumns packed with 1,4-di(methacryloyloxymethyl)naphthalene–divinylbenzene copolymer and Amberlite XAD-4 were used. In order to compare the sorption properties of these polymeric sorbents, the recoveries and breakthrough volumes of phenol, *p*-nitrophenol, 2,4-dinitrophenol, *o*-chlorophenol, *o*-nitrophenol, 2,4-dimethylphenol, 4-chloro-*m*-cresol, 4,6-dinitro-*o*-cresol, 2,4-dichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol were studied.

INTRODUCTION

The U.S. Environmental Protection Agency (EPA) lists eleven substituted phenols as "priority pollutants"[1]. The impact of priority pollutant phenols on the aquatic environment is subject to increasing attention. These compounds are important industrial chemicals of widespread usage. Phenols often observed in waste water are released into the environment by various industrial plants. These chemicals are generated by a number of processes, including the petroleum industry, the pulp and paper industry and in the syntheses of plastics and drugs[2–4].

Chlorinated phenols are formed in drinking water as a result of treatment. In aqueous solutions they occur in ppm concentrations in waste water to sub-ppb levels in drinking water[5,6]. Even at low concentrations, phenols have an adverse effect on the taste and odour of drinking water[7].

Owing to their high toxicity, effective methods for the determination of trace amounts of priority pollutant phenols in drinking and waste waters are needed. In order to measure low levels of phenols in water, preconcentration methods must be used before the analysis. Preconcentration can be performed by solvent extraction or by sorption on solids.

Coutts *et al.*[8] formed the acetate esters of six phenols before extraction with dichloromethane from water and gas chromatographic (GC) analysis. Hajslova *et al.*[9]

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also applied solvent extraction of chlorinated phenols before their determination in the form of different derivatives. Realini[10] determined priority pollutant phenols using high-performance liquid chromatography (HPLC). Preconcentration was based on ion-pair extraction. Bigley and Grob[11] applied solid-phase extraction to preconcentrate ten priority pollutant phenols. The detection of phenols was carried out using HPLC and post-column reaction with 4-aminoantipyrine and potassium hexacyanoferrate (III). Good recoveries of priority pollutant phenols from water solutions were obtained by Chladek and Marano[12] using bonded silica cartridges for preconcentration. HPLC analysis accompanied by on-line preconcentration of priority pollutant phenols was used by Baldwin and Debowski[13]. Tateda and Fritz[14] designed an Amberlite XAD-4 minicolumn for the preconcentration of some phenols, while Werkhoven-Goewie *et al.*[15,16] proposed styrene–divinylbenzene copolymers as an effective sorbents for this purpose.

In a previous paper, a porous copolymer of 1,4-di(methacryloyloxymethyl)-naphthalene (1,4-DMN) and divinylbenzene (DVB) was investigated as a sorbent for off-line preconcentration of chlorophenols[17]. As a continuation of studies on different applications of 1,4-DMN–DVB copolymer, this sorbent was used for the preconcentration of priority pollutant phenols from water. Amberlite XAD-4 was used for comparison purposes.

EXPERIMENTAL

Apparatus

A Liquochrom Model 2010 liquid chromatograph (Labor, Budapest, Hungary) equipped with an injection valve with a sample loop of 20 μl, a variable-wavelength UV detector and a 250 mm × 4 mm I.D. LiChrosorb RP-18 (10 μm) column was used. For the determination of ten of the priority pollutant phenols acetonitrile–10^{-3} *M* phosphoric acid (30:70, v/v) was used as the mobile phase, but for pentachlorophenol acetonitrile–10^{-3} *M* phosphoric acid (80:20, v/v) was used. A flow-rate of 1 ml/min was used throughout the analyses. Detection was performed at 220 nm. Quantitation of the chromatograms was based on peak heights using calibration graphs.

Chemicals

Analytical-reagent grade chemicals were used. *o*-Chlorophenol, 2,4-dinitrophenol, 2,4-dimethylphenol, 2,4-dichlorophenol, 4-chloro-*m*-cresol, 4,6-dinitro-*o*-cresol and 2,4,6-trichlorophenol were purchased from Merck (Darmstadt, F.R.G.), phenol, *o*-nitrophenol, *p*-nitrophenol, methanol, anhydrous phosphorus pentoxide and 85% phosphoric acid from POCh (Gliwice, Poland) and pentachlorophenol from Koch-Light (Colnbrook, U.K.). Owing to the small transmission of acetonitrile (Laborchemie, Apolda, G.D.R.) at 220 nm it was preliminary distilled from over anhydrous phosphorus pentoxide. Doubly distilled water was used for the preparation of mobile phases and solutions for the recovery studies.

The sample of 1,4-DMN–DVB copolymer (0.04–0.05 mm) whose properties were described in a previous paper[17] was used. Amberlite XAD-4 (Rohm and Haas, Philadelphia, PA, U.S.A.) was ground and sieved to 0.04–0.05 mm and purified according to the procedure described by Junk *et al.*[18].

Recovery studies

For the preconcentration of priority pollutant phenols from water, laboratory-made cartridges and a simple vacuum manifold, as described previously[17,19], were used. The weight of both sorbents (1,4-DMN–DVB and Amberlite XAD-4) was 200 mg. The dimensions of the sorbent beds in the dry state were length 10 mm and I.D. 9 mm. The volumes of the bed were about 1 ml. The Amberlite XAD-4 bed swelled to about 1.5 ml in methanol during the regeneration and elution steps. The volume of the 1,4-DMN–DVB bed did not change significantly.

Before sampling, each minicolumn was conditioned with 10 ml of methanol using a vacuum manifold and water aspirator, then 5 ml of doubly distilled water were added to prepare the surface of the sorbent for adsorption.

Water samples were prepared from a methanolic stock solution containing 20 μg/ml of each phenol by dilution with doubly distilled water to 0.4 μg/ml. Different volumes of these water samples were sucked through the minicolumn immersed in the sample and connected by PTFE tubing to the water aspirator. After the sample had passed through the minicolumn, the latter was installed in the vacuum manifold and 1 ml of doubly distilled water was flushed through it. The vacuum was maintained for 5 min in order to dry the sorbent bed. Priority pollutant phenols were then eluted into a collection tube with three 500-μl aliquots of methanol. After all the sorbates had eluted from the minicolumn, each sample was diluted with methanol to 2 ml or to a multiple of this volume.

The eluate in the collection tube was analysed directly or capped and stored in a freezer. Volumes of 20 μl of preconcentrated samples were injected into the liquid chromatograph. A standard phenol solution (20 μg/ml) was also injected into the chromatograph under the same conditions.

The percentage recovery of priority pollutant phenols was calculated directly from a comparison of peak heights. Recoveries were calculated as mean values of three analyses.

Preconcentration of pentachlorophenol was performed separately by the same procedure.

RESULTS AND DISCUSSION

The problem of the determination of priority pollutant phenols has been a subject of several papers[10,11,20,21]. Recovery studies require complete separation of the peaks of the standard compounds and large peak areas to minimize errors. Complete separation of ten priority pollutant phenols with the exception of pentachlorophenol was achieved using acetonitrile–10^{-3} *M* phosphoric acid (30:70, v/v) as the mobile phase as proposed by Realini[10]. The addition of phosphoric acid to the mobile phase was necessary to prevent peak tailing due to ionized phenols. Comparable peak heights of phenols were attained at a wavelength of 220 nm. The separation of ten priority pollutant phenols is shown in Fig. 1.

In order to preconcentrate priority pollutant phenols from water solutions, minicolumns packed with 1,4-DMN–DVB copolymer or the commonly used Amberlite XAD-4 were applied. 1,4-DMN–DVB porous polymer proved to be an effective sorbent for the preconcentration of various chlorophenols[17].

Table I gives the recoveries of priority pollutant phenols on these materials from

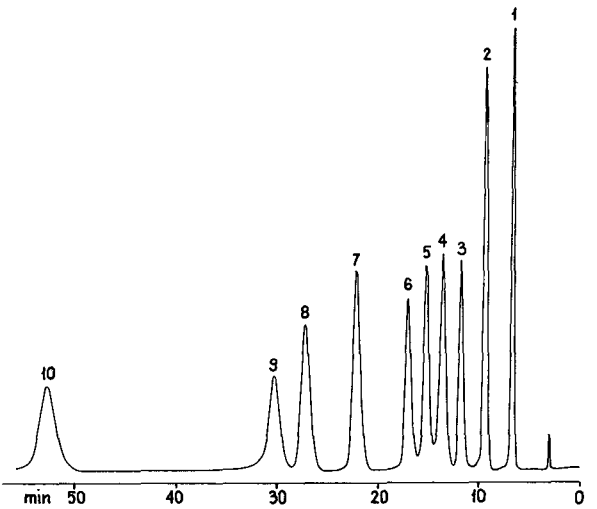

Fig. 1. Chromatogram of a standard mixture of ten priority pollutant phenols. Eluent, acetonitrile–10^{-3} *M* phosphoric acid (30:70, v/v); detection, 220 nm at 0.1 a.u.f.s. Peaks: 1 = phenol; 2 = *p*-nitrophenol; 3 = *o*-chlorophenol; 4 = 2,4-dinitrophenol; 5 = *o*-nitrophenol; 6 = 2,4-dimethylphenol; 7 = 4-chloro-*m*-cresol; 8 = 2,4-dichlorophenol; 9 = 4,6-dinitro-*o*-cresol; 10 = 2,4,6-trichlorophenol.

100 ml of aqueous solutions containing 0.4 μg/ml of each compound. It can be seen that with the exception of 2,4-dinitrophenol, the 1,4-DMN–DVB copolymer gives yields of about 100%. With Amberlite XAD-4, the recoveries of 2,4-dinitrophenol and 4,6-dinitro-*o*-cresol are not quantitative. For other compounds, recoveries with a sample volume of 100 ml are almost identical with the Amberlite XAD-4 and 1,4-DMN–DVB copolymer columns.

TABLE I

COMPARISON OF RECOVERIES OF PRIORITY POLLUTANT PHENOLS ON THE INVESTIGATED SORBENTS FOR 100-ml SAMPLES OF FORTIFIED WATER, AND BREAKTHROUGH VOLUMES

Phenol	*Recovery (%)*		*Breakthrough volume (ml)*	
	1,4-DMN–DVB	*Amberlite XAD-4*	*1,4-DMN–DVB*	*Amberlite XAD-4*
Phenol	100.4	100.6	200	200
2,4-Dinitrophenol	67.2	79.8	50	< 100
o-Chlorophenol	99.6	103.8	600	600
p-Nitrophenol	102.2	102.7	600	500
o-Nitrophenol	102.8	99.2	600	1200
2,4-Dimethylphenol	100.4	101.8	600	1200
4,6-Dinitro-*o*-cresol	102.5	92.1	800	1200
4-Chloro-*m*-cresol	101.0	102.6	800	1800
2,4-Dichlorophenol	99.2	98.3	1300	2000
2,4,6-Trichlorophenol	99.4	99.0	1300	2000
Pentachlorophenol	102.1	101.3	2000	2600

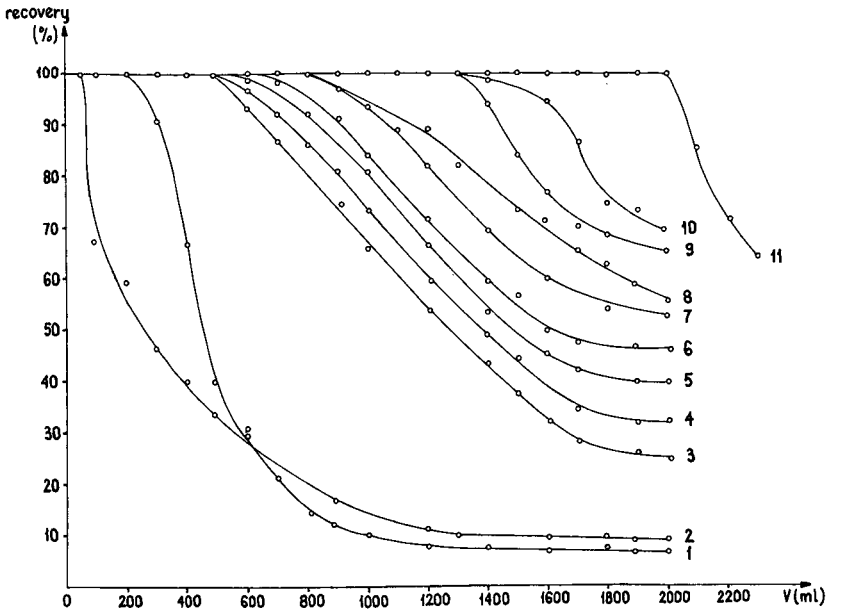

Fig. 2. Recovery (%) of (1) phenol, (2) 2,4-dinitrophenol, (3) *o*-chlorophenol, (4) *p*-nitrophenol, (5) *o*-nitrophenol, (6) 2,4-dimethylphenol, (7) 4,6-dinitro-*o*-cresol, (8) 4-chloro-*m*-cresol, (9) 2,4-dichlorophenol, (10) 2,4,6-trichlorophenol and (11) pentachlorophenol as a function of the sample volume (V). Conditions: minicolumn with 1.4-DMN–DVB porous copolymer; sampling rate, *ca.* 20 ml/min; concentration of phenols 0.4 μg/ml in water.

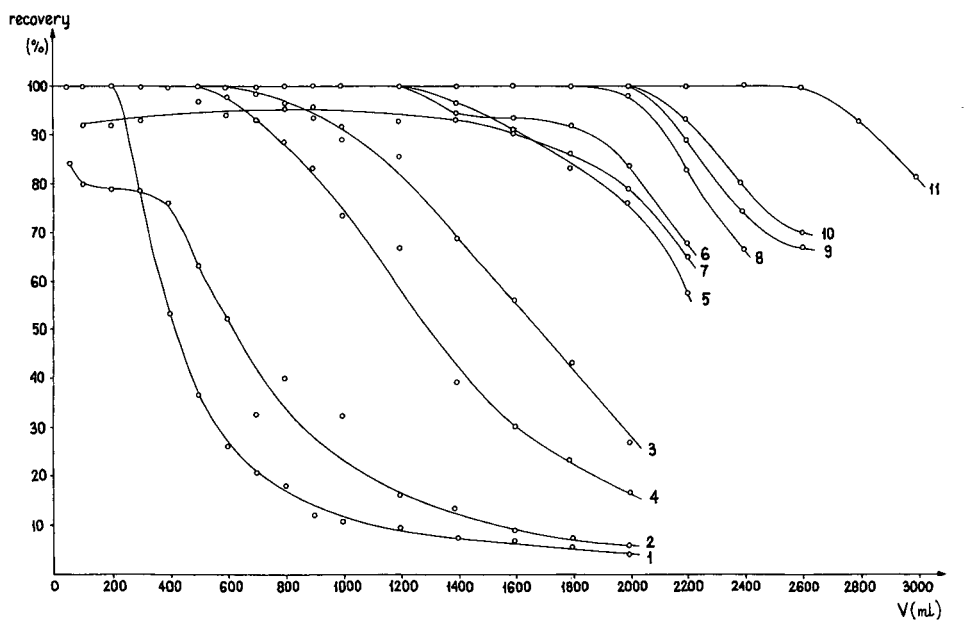

Fig. 3. Recovery (%) of phenols as a function of the sample volume (V) for Amberlite XAD-4 minicolumn. Conditions as in Fig. 2.

In order to check the applicability of porous polymers for the preconcentration of priority pollutant phenols from water solutions the breakthrough volumes were determined. Figs. 2 and 3 show the relationships between recovery and sample volume for priority pollutant phenols solutions contaning 0.4 μg/ml of each compound. It can be seen that for some phenols the same volumes can be applied before breakthrough occurs. For both porous polymers the breakthrough volume for phenol is 200 ml and for *o*-chlorophenol and *p*-nitrophenol about 500–600 ml.

For highly substituted phenols the breakthrough volumes on Amberlite XAD-4 are generally greater than those on 1,4-DMN–DVB copolymer. This phenomenon is especially visible for 2,4-dichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol. Probably the substituted chlorophenols show a greater affinity for Amberlite XAD-4 than for 1,4-DMN-DVB copolymer. Additionally, the specific surface area of Amberlite XAD-4 is about three times larger than that of 1,4-DMN–DVB and its sorption properties should be better. On the other hand, 1,4-DMN–DVB copolymer has a weakly polar character owing to the presence of ester groups in its skeleton and it probably interacts more strongly than Amberlite XAD-4 with polar compounds.

The above results suggest that in spite of the greater breakthrough volumes for highly substituted chlorophenols obtained on Amberlite XAD-4, the retention characteristics of the two polymers are similar. However, 1,4-DMN–DVB copolymer has the advantage over Amberlite XAD-4 that it does not shrink or swell with changes in the nature of the eluent because of its high degree of cross-linking.

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Characterization of humified compounds by extraction and fractionation on solid polyvinylpyrrolidone

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ABSTRACT

New humification parameters are proposed for characterizing soils and organic fertilizers or amendments. They include an index, a degree and a rate of humification, all devised to assess the ratio between humified and non-humified materials. The separation of the two types of materials is essentially performed by extraction and fractionation on solid polyvinylpyrrolidone.

INTRODUCTION

Assessing quality criteria with respect to organic matter in soils, fertilizers, sludges or composts is a difficult task, and has led to controversial results. Some workers[1], for instance, suggested the organic matter extraction of soil samples with a solution of 0.1 *M* sodium hydroxide plus 0.1 *M* $Na_4P_2O_7$, but they did not apply this procedure to organic materials other than soil. Others[2] adopted parameters based on visible spectral properties (*e.g.*, on the degree of aromaticity), but found that the nominal molecular weight distribution of humic acids decreases with increasing degree of humification, which is the opposite of normally accepted trends. Also, the results obtained using the ratio between humic acids (HA) and fulvic acids (FA) are of uncertain interpretation because this ratio depends on many factors including even the geographical distribution of soils[3]. The E_4/E_6 ratio, widely used as a index of humification[4], depends on many factors, and the addition of a small amount of humic substances (*e.g.*, HA from leonardite) is sufficient to change the results completely. Humic substances have been defined as amorphous, polymeric, brown compounds; they do not belong to recognizable classes of organic compounds, such as polysaccharides, polypeptides or, altered lignins[5]. The procedure suggested by some workers[1] considers the alkaline extract (from soil or other organic materials) as the total humic extract, but it consists of both humic and non-humic substances. After precipitation of HA by acidification of the alkaline extract, the supernatant contains both FA and other classes of organic materials (*e.g.*, polysaccharides).

Polyamide columns have been used to retain the coloured fractions of FA[6,7], whereas Amberlite XAD-8 has been preferred for adsorbing selectively humic substances from freshwater[8,9]. This last procedure has been recommended by the International Humic Substances Society (IHSS).

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Polyvinylpyrrolidone (PVP), a cross-linked adsorbent for the chromatographic separation of aromatic acids, aldehydes and phenols[10,11], has been used to separate phenols from different organic extracts[12,13]. Insoluble PVP has been found to allow strong adsorption and good recoveries of humic substances[13].

The use of a selective extraction and fractionation on solid PVP has been suggested in order to distinghuish non-humified from humified materials in soil, dung, compost and sludge extracts[14]. This paper reports further results obtained using this procedure.

EXPERIMENTAL

Soil samples

Soil samples were air-dried, crushed to pass a 2-mm sieve according to ISSS (Italian Society of Soil Science) methods[15] and stored in plastic bags.

Samples of organic materials

Samples of organic fertilizers, amendments, composts, sewage sludges and swine slurries were dried at 40–50°C in a forced-air oven, crushed to pass a 0.5-mm sieve and stored in black plastic bags.

Preparation of the polyvinylpyrrolidone resin

About 50 g of insoluble PVP resin (Aldrich) were placed into a 1-l glass cylinder. After washing twice with tap-water and twice with distilled water, discarding the fines each time, 0.005 *M* sulphuric acid was added and the suspension was stored at room temperature.

Recommended extraction and fractionation procedure

Fig. 1 shows a scheme of the proposed extraction and fractionation procedure for organic extracts using insoluble PVP to separate non-humified materials in the so-called fulvic fractions. A standard procedure can be suggested as follows.

Place 10 g of soil sample or 2 g of organic material in a 150-ml centrifuge tube with 100 ml of 0.1 *M* sodium hydroxide plus 0.1 *M* $Na_4P_2O_7$ (NaOH + PP). Let nitrogen bubble through the solution for 2 min, then plug the tube immediately. Shake for 2 h at 160 oscillations per minute at room temperature, then centrifuge at 13000 *g* for 20 min. After centrifugation, filter through a 0.45-μm Millipore filter using a vacuum pump. Transfer 25 ml of the extract (total extract, TE) into a centrifuge tube (usually 40 ml) and acidify to pH <2 by adding a small volume (0.3–0.5 ml) of 50% sulphuric acid. Centrifuge at 5000 *g* for 20 minutes, collect and store the precipitate (apparent humic acid fraction, HA) and feed the supernatant solution onto a small column (normally a common 10-ml plastic sirynge) packed with about 4–6 cm^3 of insoluble PVP previously equilibrated in 0.005 *M* sulphuric acid. Collect the eluate in a 50-ml volumetric flask, after discarding the first 2–3 ml, elute the column with about 20 ml of 0.005 *M* sulphuric acid, then dilute to volume with 0.005 *M* sulphuric acid and store for subsequent analyses (non-retained, or non-humified fraction, NH). Elute the retained fraction, generally brown (apparent fulvic fraction, FA), with 0.5 *M* sodium hydroxide solution, discarding the first 2–3 ml, and collect in the centrifuge tube containing the HA precipitate, which redissolves. Wash the col-

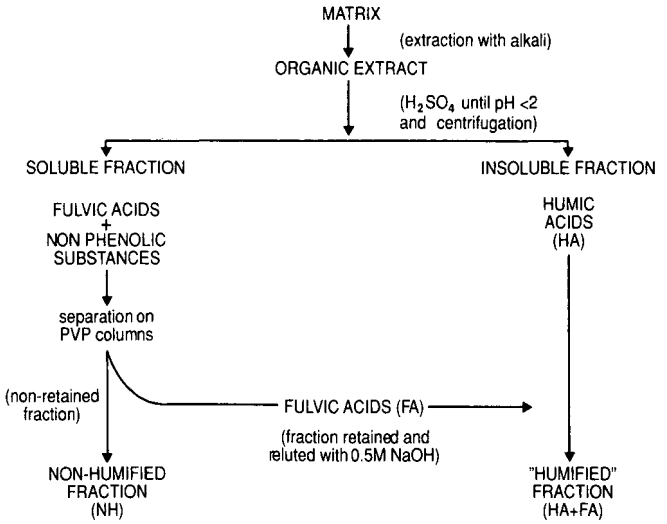

Fig. 1. Separation of humified (HA + FA) from non-humified materials (NH) by means of columns packed with insoluble PVP. Non-humified fractions are not retained on PVP; after washing with 0.005 *M* H_2SO_4 the fulvic fraction is eluted with 0.05 *M* NaOH and added to humic acids.

umn with about 20 ml of 0.5 *M* sodium hydroxide solution, transfer the combined HA + FA fractions quantitatively into a 50-ml volumetric flask and dilute to volume with 0.5 *M* sodium hydroxide solution. Store the TE, NH and HA + FA fractions in a refrigerator at 4°C for subsequent analyses.

Determination of total organic carbon

Total organic carbon (TOC) was determined according to the dichromate acid oxidation method[16]. For liquid extracts (TE, NH and HA + FA), an aliquot (normally 1–10 ml, depending on the organic carbon concentration of the sample) was used for the determination according to the same procedure.

RESULTS AND DISCUSSION

Some results achieved for selected samples of soils, organic amendments and fertilizers, composts, sewage sludges and swine slurries are given in Table I.

Three new parameters of humification are used in Table I, namely the humification index (HI)[14]:

$$HI = NH/(HA + FA)$$

i.e., the ratio between non-humified (NH) and humified compounds (HA + FA); the degree of humification (DH)[17]:

$$DH(\%) = [(HA + FA)/TEC] \cdot 100$$

TABLE I

TOTAL ORGANIC CARBON AND INDEX (HI), DEGREE (DH) AND RATE OF HUMIFICATION (HR) OF SOME SAMPLES OF SOILS, AMENDMENTS, ORGANIC FERTILIZERS, COMPOSTS, SEWAGE SLUDGES AND SWINE SLURRIES

Sample	*Total organic carbon (%)*	*Humification index (HI)*	*Degree of humification (DH) (%)*	*Humification rate (HR) (%)*
Soil 1: protorendzina, A horizon	7.4	0.14	77.5	20.9
Soil 2: mollisol, A horizon	2.5	0.18	99.0	70.9
Soil 3: histosol	19.0	0.13	82.5	22.1
Soil 4: typic xeropsamments	1.5	0.42	63.9	19.0
Soil 5: fluventic xerochrept	1.9	0.47	67.9	17.1
Soil 6: podzol, A_1 horizon	7.5	0.28	68.0	21.8
Soil 7: podzol, B_h horizon	2.7	0.17	71.0	35.4
Cow manure, well matured	18.5	0.32	76.0	8.0
Peat 1: Italy	31.1	0.30	76.9	19.3
Peat 2: Norway	48.1	0.29	77.6	22.1
Leonardite 1: U.S.A.	40.5	0.00	100.0	84.4
Leonardite 2: U.S.A.	41.3	0.05	94.9	82.7
Horn and hooves: Italy	35.6	2.87	25.9	4.2
Ground feather: Italy	47.0	34.00	2.9	0.2
Distillery washes: Italy	17.5	4.53	18.1	8.6
Leather meal: Italy	43.7	19.71	4.8	1.6
Compost: raw, from urban refuse	21.0	1.32	43.1	21.7
Compost: after stabilization	17.1	0.75	57.3	24.4
Sewage sludge: partially stabilized, from thickening bed	22.0	1.20	45.0	2.6
Sewage sludge: raw, from the outlet of anaerobic digester	26.0	2.13	31.0	2.8
Swine slurries: raw	49.5	1.20	35.7	13.9
Swine slurries: after 120 days of stabilization	30.4	0.80	48.7	12.5

i.e., the percentage of humified compounds with respect to total extracted carbon (TEC); and the humification rate (HR)[17]:

$$HR(\%) = [(HA + FA)/TOC] \cdot 100$$

i.e., the percentage of humified compounds with respect to TOC in the sample.

Table I shows that HI $\approx$ 0 (0–0.5) for humified materials (soils, organic amendments), and is much higher than 1 for non-humified materials (organic fertilizers, raw composts, sewage sludges and swine slurries). Fig. 2 shows the trend of HI during the organic matter stabilization processes in a compost from urban refuse. HI decreases continuously during humification processes, and reaches values lower than 1 at the

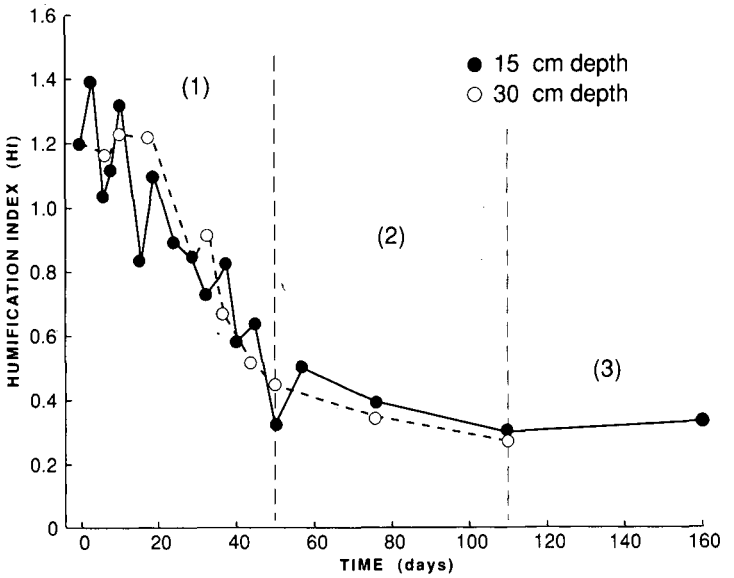

Fig. 2. Trend of the humification index (HI) during the (1) thermophilic, (2) mesophilic and (3) final phases of the organic matter stabilization process in a compost from urban refuse[23].

end of the stabilization. In other studies HI has been used to characterize organic matter from animal manures after digestion by earthworms[18] and to evaluate the maturity of organic wastes[19] and organic matter stabilization in sewage sludges[20] and swine slurries[21].

DH is higher than 60% for humified materials (soils, organic amendments), and close to 100 only for leonardites (fossil humic substances). Less humified samples (organic fertilizers, raw sewage sludges or swine slurries) show lower DH values. DH has also been used to monitor the evolution of organic matter from an organic fertil-

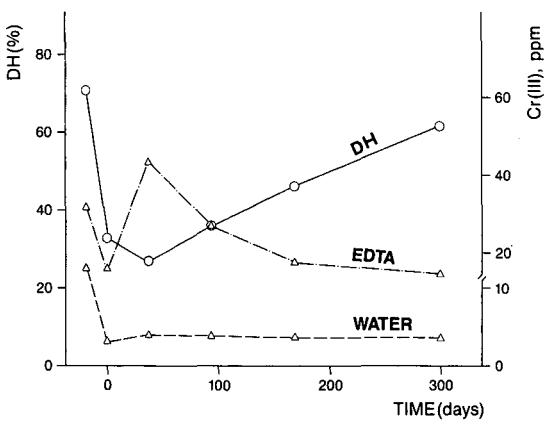

Fig. 3. Apparent degree of humification (DH) and soluble and EDTA-extractable chromium during humification of leather meal fertilizers in soil under aerobic conditions.

izer (leather meal)[22] even after application to the soil (Fig. 3) , in order to follow a possible chromium release during humification processes.

Strongly humified materials such as leonardites also show a high HR(>80%), whereas this parameter appears to be generally low for soils (except sample 2) and organic materials.

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Alternatively, a Wescan 213A conductometric detector was employed; 1 V exit was used in order to interface it to the Vista 401 data system or to the Chromato-Integrator. The latter is provided with two channels and allows a direct comparison between chromatograms obtained for the same injection by the use of the two detectors.

A Merck C_{18} Hibar LiChrospher RP-18 (5 μm) column (250 × 4.0 mm I.D.) was used.

For pH measurements, a Metrohm 654 pH meter equipped with a combined glass–calomel electrode was employed.

Chemicals

Chemicals of analytical-reagent grade and ultra-pure water from a Millipore Milli-Q system were used.

Pentylamine, heptylamine and octylamine were obtained from Fluka and salicylic acid and all other reagents from Carlo Erba.

Solutions to be used as eluents, namely pentylamine salicylate, heptylamine salicylate, octylamine salicylate and octylamine orthophosphate, were prepared by dissolving a weighed amount of the amine in water and adjusting the pH to 6.3 ± 0.4 with salicylic or orthophosphoric acid[1–4].

In order to condition the chromatographic system, eluent was passed through the column until a stable baseline was obtained (not less than 1 h). Eluent solutions were prepared freshly each third day.

The reproducibility of measurements was very good for sequential measurements and slightly poorer for different eluent preparations and column conditionings. Average data and reproducibilities of the retention data listed in Tables I–IV were calculated for different preparations.

Between uses, the column was regenerated by passing water–methanol (1:1, v/v). No particular decrease in the column lifetime was observed with respect to its use in other chromatographic techniques.

RESULTS AND DISCUSSION

Optimum conditions for the simultaneous determination of anionic species and amines were established. Parameters that were previously shown[2] to affect retention, such as the alkyl chain length of the eluent, eluent flow-rate and the stationary phase packing, were varied systematically and the results compared (Tables I–IV). UV and conductometric detectors were employed. Reasonable retention times were obtained for both amines and anions by the use of a LiChrospher RP-18 column, which was subsequently adopted throughout.

It can be concluded that the alkyl chain length of the ion-interaction reagent has the greatest influence on retention. This effect differs when the analytes are anions or amines, an increase of the alkyl chain length leading to a greater retention of anions and a lower retention of amines. The effect, which has been observed previously[4], holds for all the investigated systems.

It follows that, when the resolution between the components of a mixture needs to be improved, an eluent must be chosen that is characterized by a longer alkyl chain when dealing with anions and by a shorter chain in the analysis of amines. Thus, as an

TABLE I

RETENTION TIMES OF SOME NITROGEN-CONTAINING SPECIES

Chromatographic conditions: stationary phase, LiChrospher RP-18 (5 μm); ion-interaction reagent, pentylamine salicylate (0.0050 M) at different flow-rates.

Species	*Retention time (min) ± S.D. (n ⩾ 4)*				
	Flow-rate (ml min^{-1})				
	0.8	*1.0*	*1.2*	*1.5*	*2.0*
Ammonium	3.1 ± 0.3				
Hydrazine	3.4 ± 0.3				
Hydroxylamine	3.4 ± 0.3				
Methylamine	3.7 ± 0.3	3.3 ± 0.3	2.2 ± 0.3	2.1 ± 0.3	
Ethylamine	4.6 ± 0.3	3.8 ± 0.3	2.7 ± 0.3	2.5 ± 0.3	
Propylamine	7.2 ± 0.3	6.0 ± 0.3	4.5 ± 0.3		
tert.-Butylamine	12.7 ± 0.4	10.4 ± 0.3	7.2 ± 0.3	6.0 ± 0.3	4.7 ± 0.3
sec.-Butylamine	14.2 ± 0.4	11.6 ± 0.2	8.5 ± 0.3	8.0 ± 0.3	5.5 ± 0.3
n-Butylamine	16.8 ± 0.5	13.6 ± 0.4	9.7 ± 0.3		6.4 ± 0.3
neo-Pentylamine	34.9 ± 0.6				

TABLE II

RETENTION TIMES OF SOME NITROGEN-CONTAINING SPECIES

Chromatographic conditions: stationary phase, LiChrospher RP-18 (5 μm); ion-interaction reagent, octylamine salicylate (0.0050 *M*) at different flow-rates.

Species	*Retention time (min) ± S.D. (n ⩾ 4)*			
	Flow-rate (ml min^{-1})			
	0.8	*1.2*	*1.5*	*2.0*
Ammonium	2.0 ± 0.2			
Hydrazine	2.4 ± 0.2			
Hydroxylamine	2.4 ± 0.3			
Ethylamine	2.2 ± 0.3	1.5 ± 0.3		
Thiourea	2.3 ± 0.3			
Propylamine	3.3 ± 0.3	1.8 ± 0.3		
Butylamines	3.5 ± 0.6	3.0 ± 0.5		
p-Phenylendiamine	4.3 ± 0.3			
Benzylamine	5.5 ± 0.3			
Pentylamines	6.5 ± 0.4	4.4 ± 0.3	3.4 ± 0.3	2.2 ± 0.3
Nitrites	7.7 ± 0.4			
Nitrates	9.0 ± 0.3			
Hexylamine	12.7 ± 0.4			

TABLE III

RETENTION TIMES OF PENTYLAMINE ISOMERS

Chromatographic conditions: stationary phase, LiChrospher RP-18 (5 μm); ion-interaction reagent, heptylamine salicylate (0.0050 *M*) at different flow-rates.

Isomer	*Retention time (min) ± S.D. (n ≥ 4)*	
	Flow-rate (ml min^{-1})	
	0.8	*1.0*
2-Methyl-2-butylamine	7.0 ± 0.4	5.4 ± 0.3
2,2-Dimethylpropylamine	7.7 ± 0.4	6.0 ± 0.3
1,2-Dimethylpropylamine	7.9 ± 0.4	6.2 ± 0.3
3-Pentylamine	8.1 ± 0.4	6.5 ± 0.3
2-Pentylamine	9.0 ± 0.4	6.8 ± 0.4
2-Methylbutylamine	9.3 ± 0.4	7.2 ± 0.4
n-Pentylamine	11.5 ± 0.4	8.9 ± 0.4

TABLE IV

RETENTION TIMES (min) OF SOME NITROGEN-CONTAINING SPECIES

Chromatographic conditions: stationary phase, LiChrosopher RP-18 (5 μm); ion-interaction reagent, octylamine orthophosphate (0.0050 *M*) at different flow-rates.

Species	*Retention time (min) ± S.D. (n ≥ 4)*		
	Flow-rate (ml min^{-1})		
	0.8	*1.0*	*2.0*
Propylamine	2.7 ± 0.2	2.2 ± 0.2	
n-Butylamine	3.0 ± 0.3	2.6 ± 0.3	
Thiourea	3.5 ± 0.3	2.7 ± 0.3	1.4 ± 0.2
n-Pentylamine	5.5 ± 0.4	3.7 ± 0.3	2.2 ± 0.3
Hexylamine	10.2 ± 0.4	8.2 ± 0.4	4.3 ± 0.2
Nitrites	12.5 ± 0.4	10.3 ± 0.3	5.5 ± 0.3
Nitrates	16.9 ± 0.5	14.4 ± 0.5	7.6 ± 0.3

example, when octylamine salicylate is used as the ion-interaction reagent, *tert.-, sec.-* and *n*-butylamines show the same retention time and cannot be separated. However, separation can be achieved by the use of pentylamine salicylate, as shown in Fig. 1, recorded for a mixture of ethylamine, propylamine and the three butylamine isomers.

A similar trend was observed in the separation of pentylamine isomers. This separation is impossible when using octylamine salicylate as the ion-interaction whereas with heptylamine salicylate the separation of six of the seven isomers is possible (Table III).

If the retention times obtained for the butylamine and pentylamine isomers are compared, it is seen that the greatest retentions correspond to the normal isomer. It can be argued that the alkyl chain length, more than the molecular structure, determines retention. This different behaviour of anions and amines can be interpreted

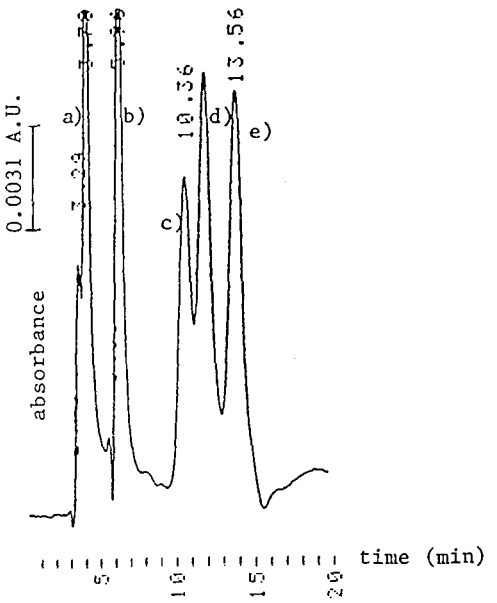

Fig. 1. Separation of a mixture of (a) ethylamine, (b) propylamine, (c) *tert.*-butylamine, (d) *sec.*-butylamine and (e) *n*-butylamine (50.0 ppm each). Injection volume: 100 μl. Stationary phase: LiChrospher RP-18 (5 μm). Ion-interaction reagent: pentylamine salicylate (0.0050 *M*). Flow-rate: 1.0 ml min^{-1}. Spectrophotometric detection at 254 nm.

through the mechanisms that explain retention[5–7] in this chromatographic technique. The eluent flowing through the stationary phase is adsorbed on the column as an ion pair, modifying the original packing and inducing a so-called "dynamic functionalization" of the column. It is generally accepted that, when active sites are still available on the stationary phase, the extent of "functionalization" increases with increase in the analytical concentration of the ion-interaction reagent and with increasing length of its alkyl chain. Greater "functionalization" generally involves increased interactions between the analyte and the stationary phase so that, if other effects do not intervene, greater retention times are to be expected. This is the situation observed for anions.

The behaviour of amines, on the other hand, is only apparently different and can be explained as follows. When amines are injected, the species that forms between the amine and the anion of the eluent is physico-chemically very similar to the ion pair of the eluent already adsorbed on the stationary phase and so is able to compete with it for the stationary phase.

These considerations also explain why only amines characterized by alkyl chains shorter than that of the eluent can be analysed. Those with longer chains do not elute in reasonable times, probably because they are more retained on the column than the "functionalizing" eluent itself.

It is concluded that the more similar are the chain lengths, the more comparable are the affinities for the stationary phase and the greater is the competition. It follows

that retention increases as the difference in the alkyl chain lengths of the analyte and the eluent decreases and *vice versa*.

These considerations and the data in Tables I–IV permitted the optimum chromatographic conditions for the separation of mixtures of interest to be established.

Figs. 2 and 3 show typical chromatograms recorded for a mixture containing propylamine, *n*-butylamine, benzylamine, nitrites and nitrates. The conditions were LiChrospher RP-18 (5 μm) as stationary phase and octylamine salicylate flowing at 0.8 ml/min as the ion-interaction reagent, with conductometric detection (Fig. 2) and spectrophotometric detection at 254 nm (Fig. 3). Owing to the molar absorptivity of eluent at this wavelength [$\varepsilon_{\text{salicylate}} = (3.08 \pm 0.02) \cdot 10^2$ l cm^{-1} mol^{-1}], nitrites and nitrates, which are transparent, appear as negative peaks and can be easily identified and resolved from amines, which under these conditions give positive peaks. It is worth remembering that transparent amines can also be detected spectrophotometrically, owing to the formation of ion pairs, *i.e.*, the corresponding aminium salicylates. According to the retention mechanism, in this form amines are retained and eluted so that the observed absorbance is due to high absorptivity of the salicylate anion.

The comparison between the behaviour of eluents has so far concerned the lipophilic cationic portion. In order to evaluate the possible role of the anion of the eluent, the use of octylamine orthophosphate was examined with constant stationary phase characteristics. Some typical retention times are given in Table IV.

As can be observed, with respect to the use of octylamine salicylate (Table I), greater retentions of anions were obtained and almost the same retentions for amines.

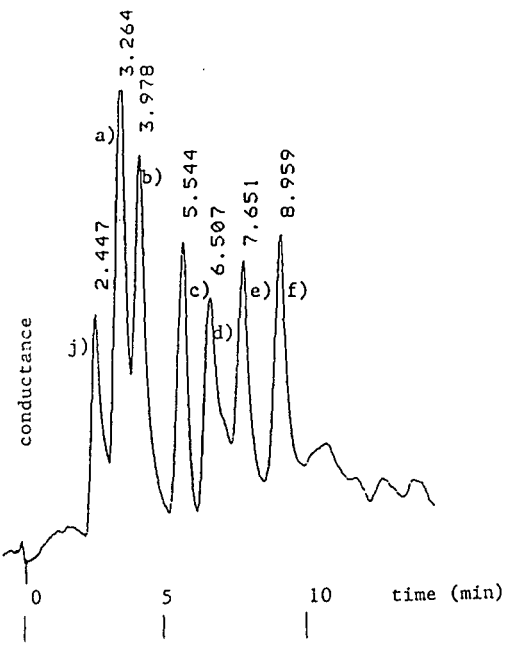

Fig. 2. Separation of a mixture of: (a) propylamine, (b) *n*-butylamine, (c) benzylamine, (d) pentylamine, (e) nitrites and (f) nitrates. Concentrations in the mixture: amines 50.0 ppm each, nitrite and nitrates 10.0 ppm each. Injection volume: 100 μl. Stationary phase: LiChrospher RP-18 (5 μm). Ion-interaction reagent: octylamine salicylate (0.0050 *M*). Flow-rate: 0.8 ml min^{-1}. Conductometric detection.

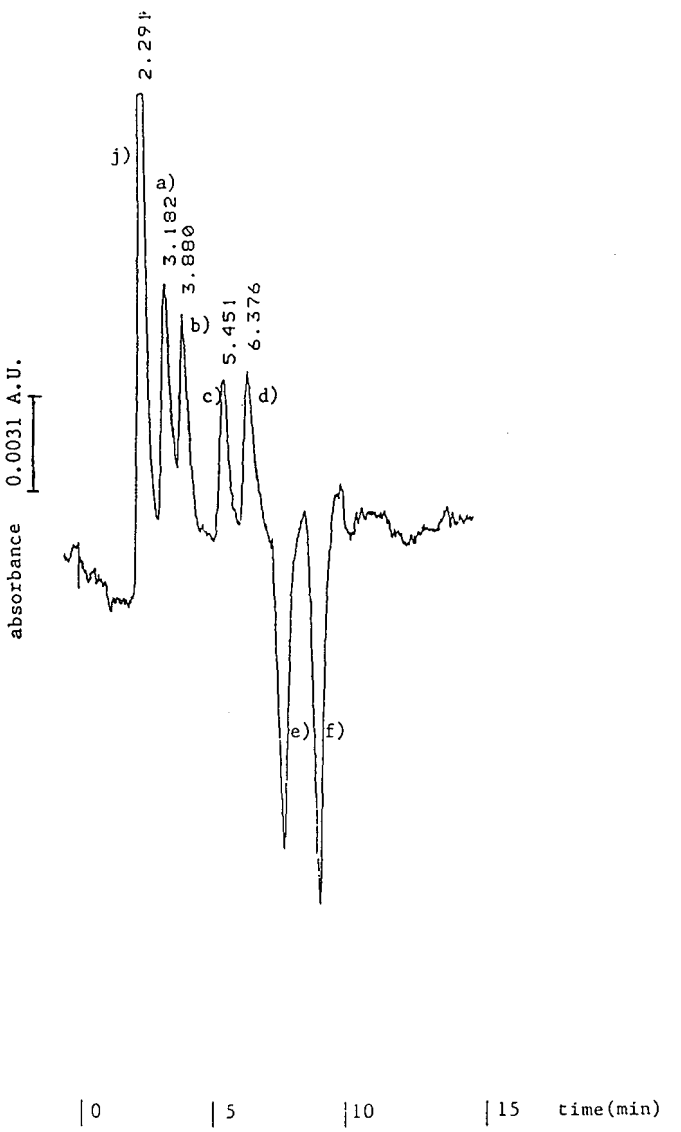

Fig. 3. Separation of the mixture as in Fig. 2, with 50.0 ppm of each components in the mixture. Conditions as in Fig. 2 except spectrophotometric detection at 254 nm.

This result agrees well with the described mechanism, according to which injected amines form an ion pair with the anion of the eluent and in this form are adsorbed and retained through direct interactions with the stationary phase. The results confirm that these interactions would mainly interest the aminium portion rather than its counter anion.

The sensitivity is about the same order for amines and nitrates with octylamine salicylate as eluent, with both conductometric and UV detection (Figs. 2 and 3),

whereas with the use of octylamine ortho-phosphate as eluent with detection at 210 nm, sensitivities about a 1000-fold greater are obtained for nitrites and nitrates with respect to amines, as shown in Fig. 4, which is a chromatogram recorded with a mixture containing 100.0 ppm each of butylamine, pentylamine and hexylamine, 0.20 ppm of nitrites and 0.20 ppm of nitrates. These results, in addition to demonstrating the exceptionally good sensitivity in the analysis of nitrites and nitrates, agree with the retention mechanism concerning amines. The low sensitivity in their analysis corresponds well with the low molar absorptivity at 210 nm shown by the investigated amines, the ortho-phosphate which forms an ion pair with the amine being almost transparent.

The high absorptivities of nitrites and nitrates under these conditions can find applications of practical interest, *e.g.*, in samples of environmental interest such as drinking, river and waste waters. The determination of nitrites and nitrates is in fact possible in the presence of all those potential interferents which do not absorb at 210 nm or are characterized by greater retentions.

The high concentration of chlorides in sea water makes the evaluation of other species generally very difficult. It was shown (Fig. 5) that, by using octylamine phosphate as the ion-interaction reagent and with spectrophotometric detection at 230

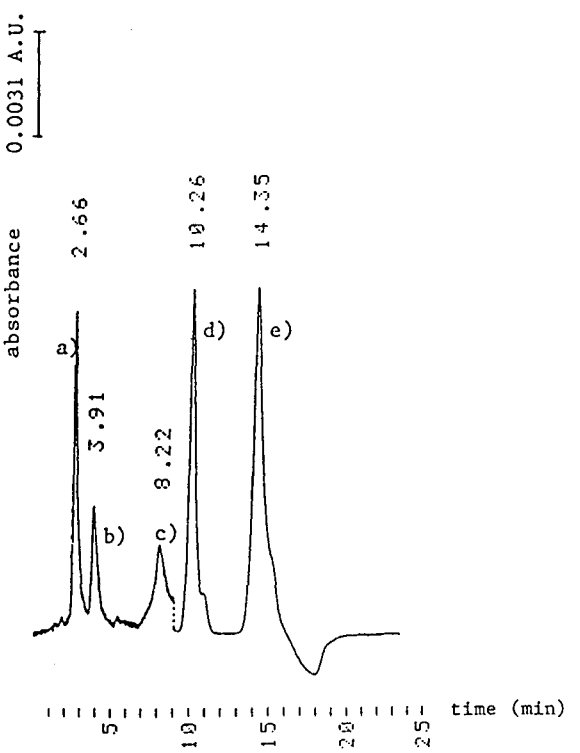

Fig. 4. Separation of a mixture of (a) n-butylamine (100 ppm), (b) *n*-pentylamine (100 ppm), (c) n-hexylamine (100 ppm), (d) nitrites (0.20 ppm) and (e) nitrates (0.20 ppm). 100 μl injected. Stationary phase: LiChrospher RP-18 (5 μm). Ion-interaction reagent: octylamine orthophosphate (0.0050 *M*). Flow-rate: 1.0 ml min^{-1}. Spectrophotometric detection at 210 nm.

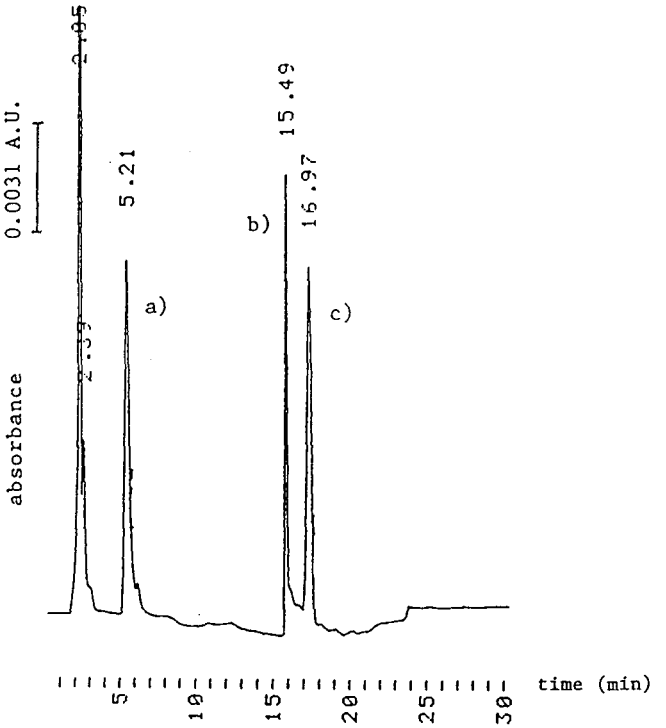

Fig. 5. Chromatogram of Venice lagoon water with 0.50 ppm of 1,4-phenylenediamine added. Peaks: (a) added 1,4-phenylendiamine; (b) unidentified; (c) nitrates (0.20 ± 0.05 ppm). Stationary phase: LiChrospher RP-18 (5 μm). Ion-interaction reagent: octylamine orthophosphate (0.0050 *M*). Flow-rate: 0.8 ml min^{-1}. Spectrophotometric detection at 230 nm.

nm, it is possible to determine in sea water all the species characterized by high molar absorptivities, even in the presence of such a large amount of chlorides, which are transparent at this wavelength. The same analysis with conductometric detection shows only a very large positive peak due to chlorides, which precludes any separation in the retention time window between 2 and 16 min.

It was observed that chlorides when present in so high amounts as in sea water (even if not detected spectrophotometrically) can shift the retention times of anions characterized by similar retention towards times much higher than those shown by the same anions in standard aqueous solutions. Accurate studies of matrix effects are necessary in order to perform a reliable and complete analysis of lagoon water for the accurate identification and determination of nitrites, iodides and bromides, which are probably responsible for the peak at about 15 min in Fig. 5. In contrast, the peak at about 17 min was identified as corresponding to nitrates, which were also determined. Their concentration was 0.20 ± 0.05 ppm.

The determination of aromatic amines in lagoon water is possible under these conditions. Fig. 5 shows the chromatogram recorded for a sample from Venice lagoon to which 0.50 ppm of 1,4-phenylendiamine had been added. This addition did not cause any shift or modification of the pre-existing signals already discussed.

In conclusion, the proposed method for the simultaneous determination of amines, nitrites and nitrates seems to be very advantageous. In particular, the use of octylamine orthophosphate offers the advantage of exceptionally good sensitivity for nitrite and nitrate anions. The accuracy and reproducibility are more than satisfactory and the analysis times are short. In contrast to other proposed techniques[8], no derivatization or pretreatment procedures are required.

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Liquid chromatography of liquid substances characterized by limited solubility in the eluent

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ABSTRACT

A new version of liquid chromatography using component distribution between immiscible phases of a liquid sample and a liquid eluent has been developed. The method, called chromaextraction, is performed on a low-sorbing column packing under high loading conditions, similarly to the gas chromatographic method of chromadistillation. They both differ from traditional chromatographic techniques in the lack of a sorbent, as the separated mixture itself may be regarded as the stationary liquid phase.

When one component possessing limited solubility in the eluent is eluted, the breakthrough curve forms a rectangular zone corresponding to a saturated solution of this component. Ordinary isocratic elution of a mixture leads to frontal-type partial separation. The first zone contains all components and the number of components decreases by one in subsequent next zone, the last zone containing only the least soluble single solute.

To obtain a complete separation in adjacent zones of components, a restriction mode of operation may be applied. This is achieved by preliminary injection of an additional component, possessing the highest (but still limited) solubility in the eluent. Extraction of solutes arising on the restrictor rear boundary and further zone boundaries results in the separation of single solute zones, in order of decreasing solubility.

It was found, that the chromatographic process under overloading conditions with limited solubilities of substances is similar on different supports, owing to the occurrence of sample–eluent demixing. The application of a restrictor allows the separation of large samples to be improved and dilute solutions to be enriched up to saturation during separation on a sorbent.

Experiments were performed using PTFE microcolumns, packed with nonporous stainless-steel powder or with octadecyl silica. Two- and three-component mixtures of nitroalkanes were eluted with water and nitromethane was used as the restrictor. Separations of benzene, toluene and halobenzenes, eluted with water–

methanol, are other examples of the successful application of the restrictor mode of operation

INTRODUCTION

The wide application of chromatography to the separation and analysis of mixtures is due to the diversity of chromatographic modes using different types of separation systems and modes of operation. Zhukhovitskii and co-workers[1,2] proposed an original gas chromatographic method with extreme column overloading. The method, called chromadistillation, is based on the selective distribution of components between a quasi-stationary liquid phase of a separated mixture and a mobile phase consisting of its saturated vapour. A mixture is injected onto an inert column packing and moves through the column by means of a carrier gas. Application of a negative temperature gradient or injection of an additional component (called a "restrictor"), posessing maximum volatility leads to complete separation in adjacent zones of pure substances, in order of decreasing volatility. Chromadistillation is achieved by injection of much larger samples than is possible in traditional elution chromatography. Hence this method appears to be particularly suited to preparative-scale separations and to the enrichment of impurities.

When a column packed with sorbent rather than an inert support used under overloading conditions, the amount of liquid sample is sufficiently high for distillation to play a significant role in separation. Such a mode of operation may be regarded as intermediate between the usual gas chromatography and chromadistillation[3].

It seemed to be expedient to apply the same principle in liquid chromatography[4,5]. The idea is to use a mixture itself as a quasi-stationary liquid phase. It is clear that limited miscibility of two phases must exist when using a mobile liquid phase. To satisfy this demand, a large amount of a mixture consisting of slightly soluble compounds should be injected onto the column without preliminary dissolution in the eluent or in another solvent. This differs from the usual practice in liquid chromatography and places certain restrictions on the application of standard instruments.

It should be mentioned that the problem of limited solubility of substances in the eluent has not been sufficiently studied, and it is often regarded as only a negative phenomenon. However, two chromatographic techniques are known that use limited solubility in a positive sense. The first is precipitation chromatography, proposed by Baker and Williams[16] for the separation of polymer molecules. The method is based on equilibrium between a polymer gel in the stationary phase and a saturated solution in the mobile phase. Recurrence of precipitation is effected by combination of a solvent gradient (to higher solubility) and a temperature gradient along the column (to lower solubility). In modern high-speed versions of liquid chromatography[7], a temperature gradient is not applied and precipitation is achieved by the exclusion effect.

Schwarz[8] developed a chromatographic method for the measurement of solubility in liquid–liquid systems. Initially the column is filled with a liquid substance, then a solvent is fed into the column. When the excess of the first liquid has been replaced, the remainder is held on a support as a stationary film, which is dissolved by

the mobile phase. Measurement of the stationary liquid boundary velocity permits the determination of solubility.

We have demonstrated the possibility of effecting separations under such conditions and examined the features of the elution of one-, two- and three-component mixtures from two types of packing (a low-sorbing support and octadecylsilica). The method of separation on a low-sorbing packing, in which components of the sample are extracted by a liquid eluent under chromatographic conditions, is named chromaextraction.

EXPERIMENTAL

Experiments were performed on a JASCO Familic-100-N instrument (Japan) with a Uvidec-100-III variable-wavelength UV detector. The columns were PTFE tubes (15 cm × 0.5 mm I.D.) packed with non-porous particles of stainless-steel powder (40-μm fraction) or with Finepack Sil C_{18} (10-μm fraction) with the use of a high-pressure syringe.

A syringe pump allowed the mobile phase flow-rate to be varied from 1 to 30 μl/min. A tube-type injector was used in the stop-flow mode of operation, the sample size being from 0.2 to 1.9 μl. If it was necessary to inject larger volumes of substances immiscible with the eluent, repeated injections were made.

Some experiments were performed on a Bruker LC31-B instrument equipped with a UV detector, a loop-type injector and a stainless-steel column (5 × 0.46 cm I.D.) packed with LiChrosorb RP-18 (10-μm fraction). This type of column was less suitable for our experiments owing to an undesirable increase in the column inlet pressure when substances of low solubilities were injected.

When a column packed with stainless-steel powder was used, distilled water served as the eluent and nitroalkanes were the substances to be separated. Water–methanol was used as the eluent for the reversed-phase separation of benzene, toluene and halobenzenes.

RESULTS AND DISCUSSION

Columns packed with a low-sorbing non-porous support were used to ensure that the following conditions were satisfied:

(1) The surface of the support is wettable better by the sample phase than by the eluent. When a liquid sample characterized by limited miscibility with the eluent is injected onto the column, it spreads on the column packing and a quasi-stationary liquid film is created. The thickness of the stationary liquid film is constant, as has been demonstrated by others[6].

(2) Conditions for mass transfer between the mobile and stationary phases are satisfactory. When a flow passes through the column the both phases are equilibrated to saturated concentrations.

(3) The amount of sorbate per unit layer volume is negligible at any solute concentration because the surface is inert or the surface area is very small. Only at solute concentrations close to saturation does the sorption increase abruptly. This means that one of the two liquid phases of the demixing solution precipitates onto the solid surface.

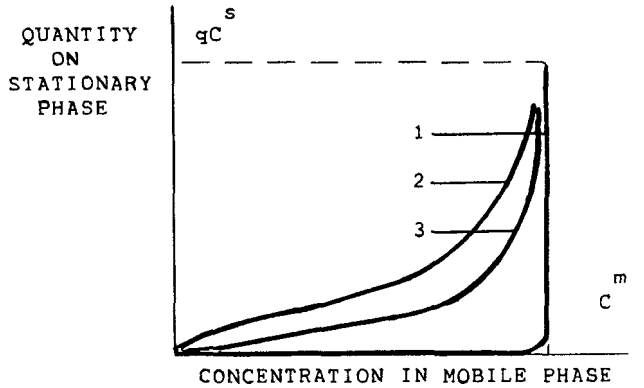

Fig. 1. Different types of single-solute adsorption isotherms from demixing solution of liquids. 1 = Extremely concave (non-sorbing wettable surface); 2 = σ-shaped; 3 = concave.

The sorption isotherm of a single component from a demixing solution on an inert wettable surface is an extremely concave curve (Fig. 1). It consists of two segments; the horizontal segment goes from zero to solubility point c_0^m and the vertical segment passes through this point and rises to qc_0^s. When a mobile phase flows through a sorbent layer, c_0^m and c_0^s correspond to the equilibrium concentrations in the mobile and stationary phases, respectively, q is the largest stationary portion of liquid and qc_0^s is the maximum amount of the component in the stationary phase.

When a dose Q of a liquid substance is injected into a packed column, which is then flushed with an eluent, it spreads over the surface of the particles and occupies a length of column that is proportional to Q (Fig. 2), the mobile and stationary phases being then equilibrated to saturation. The front boundary of the solution zone moves through the column at an eluent velocity α, if sorption from the mobile phase does not occur. The front boundary retention volume is equal to the void volume of the column. At the rear boundary of the solution zone permanent dissolution of the stationary liquid by the eluent takes place. The velocity U of the boundary can be determined from the mass balance equation:

$$\alpha c^m s^m = U(c^s s^s + c^m s^m) \tag{1}$$

where c^m and c^s are the concentrations of the mobile and stationary solutions, respectively (here and subsequently the symbol "0" is omitted) and s^m and s^s are the column sections occupied by mobile and stationary phases, respectively or

$$U = \alpha/[1 + qc^s/(1-q)c^m] \tag{2}$$

Eqn. 2 is also well known in the theory of non-linear ideal chromatography and describes the desorption boundary movement for the case of a concave isotherm. When dissolution is finished, the rear boundary velocity becomes equal to the front boundary velocity α. Hence the resulting volume V of the zone on a breakthrough curve is proportional to the sample size:

$$V = 100\, q/c^m \tag{3}$$

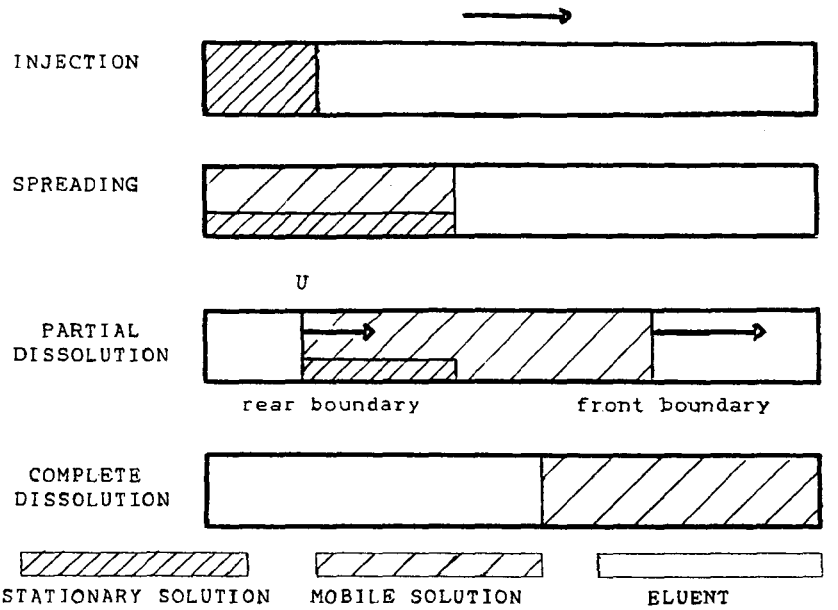

Fig. 2. Zone diagram of elution of one component from an inert packing.

where the solubility c^m is expressed in volume percent. As the concentration of the eluate is constant, the elution chromatogram has the form of a rectangular step, and the height of the step does not depend on sample size.

For experimental testing of this conclusion, some individual substances were eluted with water. In Fig. 3A the elution curves for different doses of nitroethane are depicted. Rectangular chromatograms are observed, the zone heights being identical and lengths increasing with increase in dose. Similar results were obtained when nitromethane and 2-nitropropane were eluted. If the sample size Q is known, the solute concentration generated at the column outlet can be calculated according to eqn. 3 from the length of the zone measured on the chromatogram at half of the zone height. By comparison with literature data it was found that the solute concentrations are approximately equal to the solubilities of eluted substances (9.5, 4.6 and 1.7 vol.-% for nitromethane, nitroethane and 2-nitropropane, respectively).

If the eluate concentration is known, it is possible to determine the maximum amount Q_{max} of quasi-stationary phase retained on the column packing. First the column is completely filled with a liquid substance, then another liquid (solvent) is fed into the column. When the excess of the first liquid has been removed, there is a liquid stationary film along the whole column length. Measurement of the solution zone volume on the chromatogram permits Q_{max} to be evaluated. When nitroalkanes were eluted with water this was about 25% and 15% of the void volume of the column when the flow-rate was *ca.* 0.2 and 1 mm/s, respectively.

When a column packed with octadecylsilica is used, the third condition for chromaextraction performance is not satisfied when the adsorption is high for all solute concentrations. Experiments carried out earlier[9] showed that the adsorption

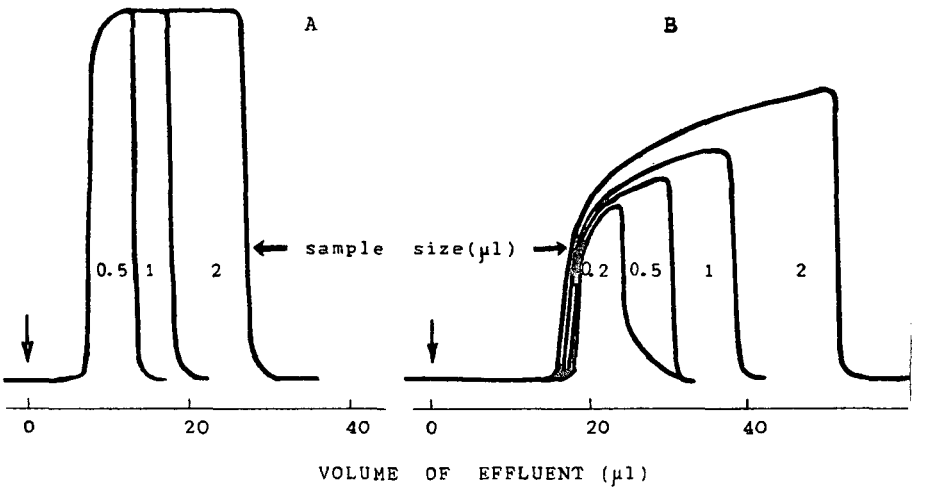

Fig. 3. Dependence of elution curve profiles on nitroethane sample size. Columns, 150 × 0.5 mm I.D.: (A) stainless-steel powder, 40 μm; (B) Finepack Sil C_{18}, 10 μm. Eluent, water at 2 μl/min; UV detection at 245 nm.

isotherms of nitroalkanes from aqueous solution on octadecylsilica are σ-shaped curves (Fig. 1, type 2). Adsorption of other substances dissolved in water–methanol was also examined, the solubilities of the sorbates being several percent. It was found that the isotherms of benzene, toluene and other aromatics are concave curves over almost the whole of the concentration interval (Fig. 1, type 3).

In accordance with the expected types of isotherms, the elution curves of nitroalkanes have a diffuse rear part when small doses are eluted (convex section of the isotherm). Further increases in the sample size result in sharpening of the peak "tail" and in dispersion of the peak front (Fig. 3B). In this way the peak shape approaches a step, with the step length and height approximating the values on chromatograms obtained using a low-sorbing packing. Hence in the case of limited solubilities in the elutent and at high loadings the solution zone concentration approaches saturation on a sorbing support.

The regularities of the elution of two-component and more complex mixtures are more complex than that of a single component. They are based on the distribution of the components in a demixing multi-component system. A binary mixture and the eluent comprise a three-component system, which may be described by equilibrium diagrams of different types. Here we do not list all possible types but only the two simplest types with limited mutual solubility of the components. In Fig. 4A neither component 1 nor 2 (mixture) is completely soluble in eluent 3. Two demixing liquid phases may exist at any ratio of the first two components. If component 1 only possesses a limited solubility, the area of demixing corresponds to a high content of this component (Fig. 4B).

When a mixture is injected into a column two phases are generated, the first being a solution of the mixture in the mobile phase and the second the mixture itself with the eluent dissolved in it (composition M_{II}, S_{II}, in Fig. 4). According to the phase diagram, the mobile phase should be enriched compared with the stationary phase by

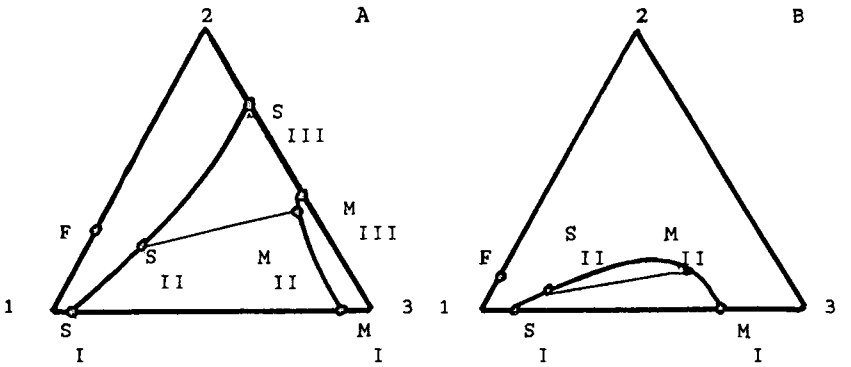

Fig. 4. Three-component solubility diagrams.

the more soluble component 2, hence an excess of component 1 remains in stationary phase. As a consequence, such an extraction process under chromatographic conditions leads to separation on zone II (mixture) and zone I (component 1) and a boundary between the zones appears (Fig. 5). The boundary velocity U_{II} and also U_I can be determined from the mass balance equation for component 1 by analogy with eqns. 1 and 2. Further, it is possible to demonstrate that $U_I < U_{II}$, but the proof is cumbersome. An elucidation of the problem with some additional assumptions may be found in the theory of chromadistillation[3].

The ordinary elution of a binary mixture by a slowly dissolving eluent from a non-sorbing packing results in separation into two zones. Three boundaries, possessing different velocities α, U_{II} and U_I are formed. The process continues until complete dissolution of the mixture occurs, then all the boundaries move with the same velocity α. At the column outlet zone II is registered first and zone I follows immediately after. In this version of partial separation the maximum sample size may be drifted over the whole column length without deterioration of separation.

Elution of binary mixtures of nitroalkanes by water was performed. The chromatograms in Fig. 6A consist of two extended zones. The height of the first depends on the composition of the initial mixture, and it may be approximated by the expression $h_1\chi_1 + h_2\chi_2$, were h_1 and h_2 are the heights of single-component zones and χ_1 and χ_2 are the molar fractions of substances in the initial mixture. The discrepancy between the measured and assumed values is not more than 10%. The heights of the

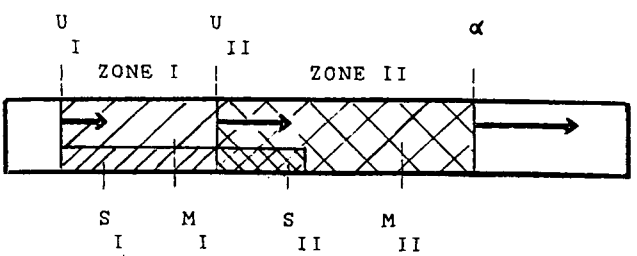

Fig. 5. Zone diagram of elution of a two-component mixture.

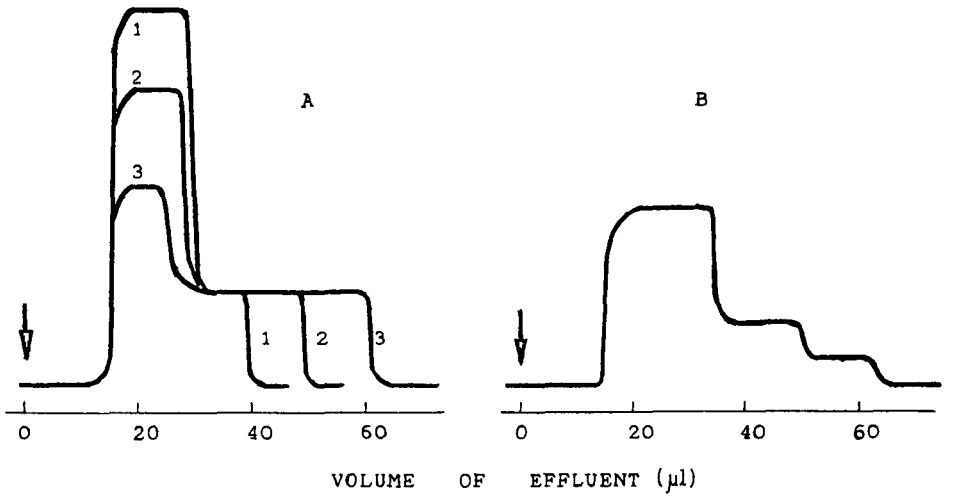

Fig. 6. Partial separation of (A) two-component and (B) three-component mixtures. Mixtures: (A) nitromethane–2-nitropropane, (1) 4:1, (2) 1:1, (3) 1:4; (B) nitroethane–2-nitropropane–2-nitrobutane, 12:4:1; sample size, 1 μl. Other conditions as in Fig. 3A.

second zone in Fig. 6A are identical and equal to the height h_1 of the less soluble component zone. In Fig. 6B a three-step chromatogram of a three-component mixture is shown. The preceding discussion confirms that the first zone on the chromatogram contains all components and the number of components decreases by one in each following zone, the last zone being a saturated solution of the least soluble compound. This type of partial separation seems to be the most similar to frontal-desorption separation.

In Fig. 7 other examples of partial separations of substances with different solubilities are given. In the first instance the binary mixture contains several percent of the less soluble component and the second is the reverse. As follows from Fig. 7A, the less soluble non-basic component forms a zone of saturated solution at the end of the chromatogram, even being at low concentration in the initial mixture. The last non-trivial result allows such a mode of operation to be recommended for the enrichment of impurites having a lower solubility than the basic component.

When an impurity component is completely soluble it forms a peak at the beginning of the chromatogram (Fig. 7B). The reason is that the concentration of this component in the mixed zone is sufficiently high for the extent of its boundary region. When a 1-butanol–acetone mixture is eluted with water, the peak height of the completely soluble acetone is proportional to its content in the initial mixture. 1-Butanol is not detectable, and therefore a negative step is registered immediately after the peak. When the concentration of acetone rises up to about 10% the separation deteriorates, and eventually complete miscibility of the sample and eluent occurs.

Some separations of compounds possessing limited solubility were executed on a sorbent packing under overloading conditions. The chromatograms in Fig. 7C show the separation of toluene and benzene (solid line) and the elution curve of toluene only (dashed line), the latter being given for comparison. A clear feature is

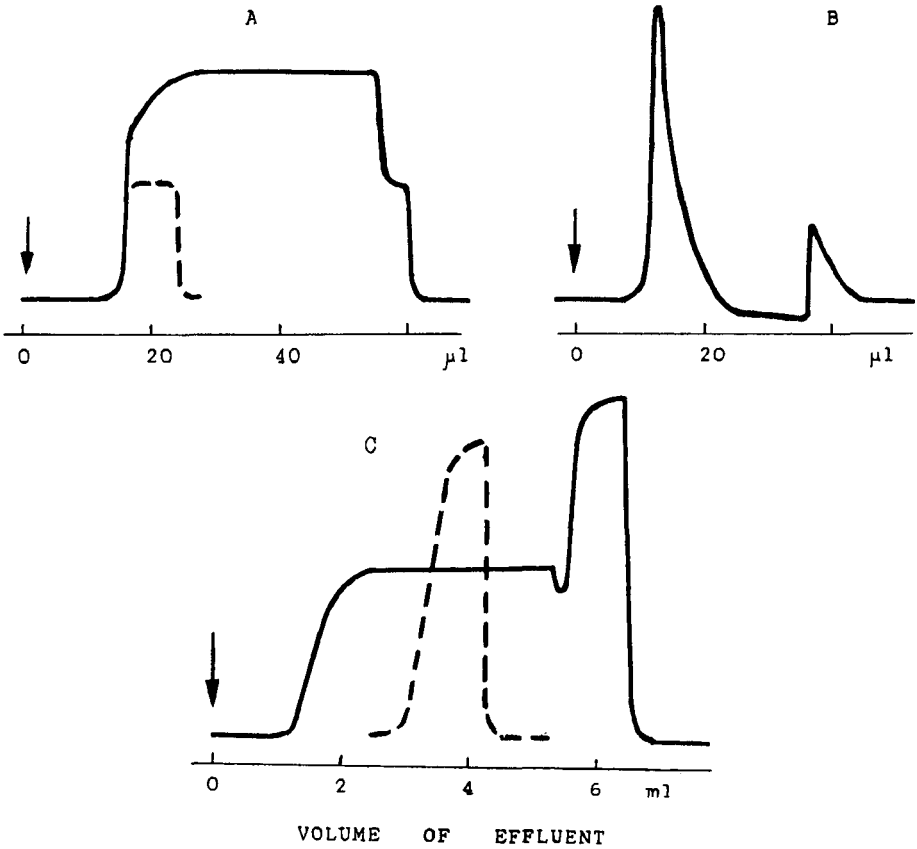

Fig. 7. Partial separation of two-component mixtures containing (A,C) a less soluble and (B) a more soluble non-basic component. Columns: (A,B) 150 × 0.5 mm I.D., stainless-steel powder, 40 μm; (C) 50 × 4.6 mm I.D., LiChrosorb RP-18, 10 μm. Eluent, (A,B) water at 2-μl/min; (C) water–methanol (30:70) at 0.5 ml/min; UV detection at (A) 245, (B) 220 nm and (C) 260 nm. Mixtures: (A) nitroethane–2-nitropropane (95:5), 1 μl; (B) acetone–1-butanol (2:98), 1 μl; (C) benzene–toluene (95:5), 100 μl.

that the less soluble non-basic component is "driven back" when the more soluble basic component is present.

This result may be explained by means of the previous discussion. We can compare separations obtained on a non-sorbing support and on a sorbent. (Fig. 7a and c) when the solubilities of the components and their ratio in the initial mixture are not very different. On both chromatograms the first elongated zone contains the most soluble basic component almost at saturation, the concentration of the other solute being low. Only behind the mixed zone does that of the less soluble component appear. The solute concentration in the last zone is close to saturation irrespective of the initial composition of the mixture. Hence it may be affirmed that if the amount of liquid sample is very large, the distribution of the components is determined to a considerable extent by liquid–liquid equilibrium and not by adsorption on the surface of the solid support.

Another interpretation of this phenomenon is that the changes in the band retention and concentration of substances are influenced by multi-component adsorption at high concentrations. The band concentration and retention increase is known as the restriction effect[3]. It is the reverse of the displacement effect[10], which leads to a decrease in band retention. Both are concerned with non-linear chromatography where the sorption isotherms are concave (restriction) or convex (displacement). Just as the displacement effect allows the displacement mode of operation to be performed, so the restricton effect may be used for execution of the restricton mode, as was done earlier in chromadistillation. In our method the least sorbed component, called the "restrictor", must possess the highest (but still limited) solubility and be injected before a mixture.

The restricton mode of operaton is applied first for the separation of a two-component mixture on a low-sorbing packing, the most soluble compound 2 being used as a restrictor. Initially a large amount of the restrictor is placed on the column as a stationary phase (Fig. 8). The eluent dissolves the stationary film and the restrictor rear boundary moves. Then a mixture of 1 + 2 is placed on the vacant part at the inlet of the column. A boundary is created between the restrictor and the mixture, and extraction of components at this boundary occurs. The velocity U_{III} of the boundary can be found from the mass balance equation of component 1:

$$U_{III} = \alpha/[1 + c^{s}_{II,1}q_{II}/(1-q_{II})c^{m}_{II,1}] \tag{4}$$

where q_{II} is a portion of stationary liquid in zone II (Fig. 8) and $c^{s}_{II,1}$ and $c^{m}_{II,1}$ are the concentrations of component 1 in the same zone in stationary and mobile phase, respectively.

At the inlet part of the column a boundary between the mixture and the less soluble component 1 appears just as in ordinary elution and its velocity is

$$U_{II} = \alpha/[1 + c^{s}_{II,2}q_{II}/(1-q_{II})c^{m}_{II,2}] \tag{5}$$

where $c^{m}_{II,2}$ and $c^{s}_{II,2}$ are the concentrations of component 2 in zone II in the mobile and stationary phase, respectively.

If the distribution factor of the less soluble component is the larger, *i.e.*, $c^{s}_{II,1}/c^{m}_{II,1} > c^{s}_{II,2}/c^{m}_{II,2}$, from eqns. 4 and 5 it follows that $U_{II} > U_{III}$ and after a period of time the boundaries coincide, so the mixed zone disappears. Hence the possibility of separation in such a mode of operation depends on several factors: column length,

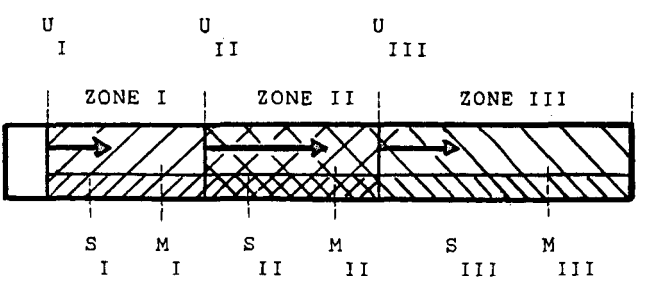

Fig. 8. Zone diagram of restriction mode of a two-component separation.

distribution factors of the components (boundary velocities), amounts of mixture and restrictor and time interval between injections of mixture and restrictor.

Earlier we assumed that the amount of mixture (sample) is suitable for occupying the part of the column vacated by the restrictor. If this is not so and the amount of mixture is greater, it is pushed forwards and a mixed zone of variable composition forms. If the amount of mixture is smaller, it is dissolved by the eluent and carried to the restrictor rear boundary. Further extraction generates a new mixture zone. Therefore, the last two situations may be converted into the former one.

In Fig. 9A the separation of binary mixtures of the same composition as in Fig. 6A is shown, with the difference that the more soluble component is used as a restrictor. All the chromatograms consist of two zones with identical heights (but different lengths), equal to the heights on the chromatograms of the individual substances, *i.e.*, the compositions of the zones c_i^m correspond to single-solute saturated solution. The absolute amount of each component i, calculated as $q_i = V_i c_i^m/100$, where V_i is the volume of the zone in a mixed chromatogram, does not differ from the known initial value by more than 10%. The restrictor need not be a component of the mixture to be separated; the only essential requirement is limited solubility of this component in eluent, but higher than that of the other components. In Fig. 9B the chromatogram of ternary mixture of nitroalkanes is shown, the restrictor not being a component of the initial mixture. The chromatogram contains four steps, each representing a zone of saturated solution of a single compound, in order of decreasing solubility. The step heights on chromatogram are equal to those of the individual substances, so the completeness of separation is confirmed.

It is possible to carry out the restriction mode of operation on a sorbent packing. This is especially useful for large sample separations. In this instance extraction of the solutes on the restrictor rear boundary is an additional factor for improving the separation process. Two- and three-component separations under overloading condi-

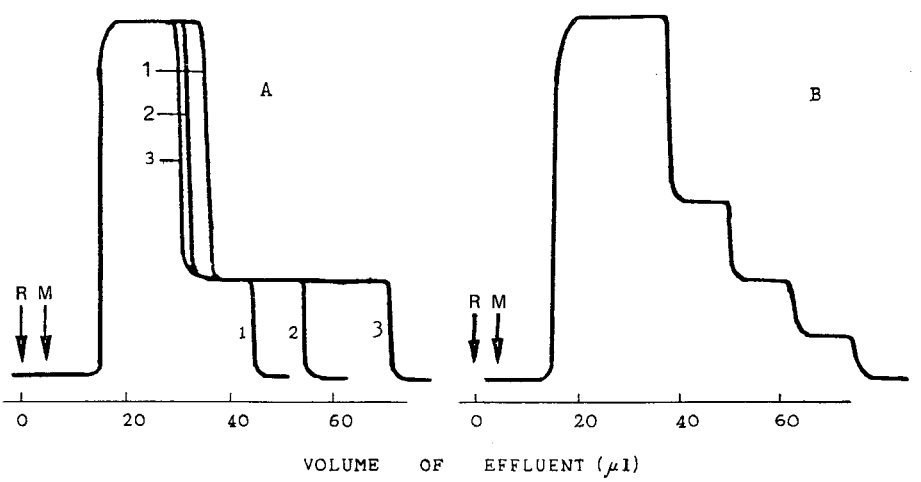

Fig. 9. Restriction mode of separation of (A) two-components and (B) three-components mixtures. Restrictor: nitromethane, (A) 2 μl, (B) 3 μl. Mixture and restrictor injections are indicated by M and R, respectively. Other conditions as in Fig. 6.

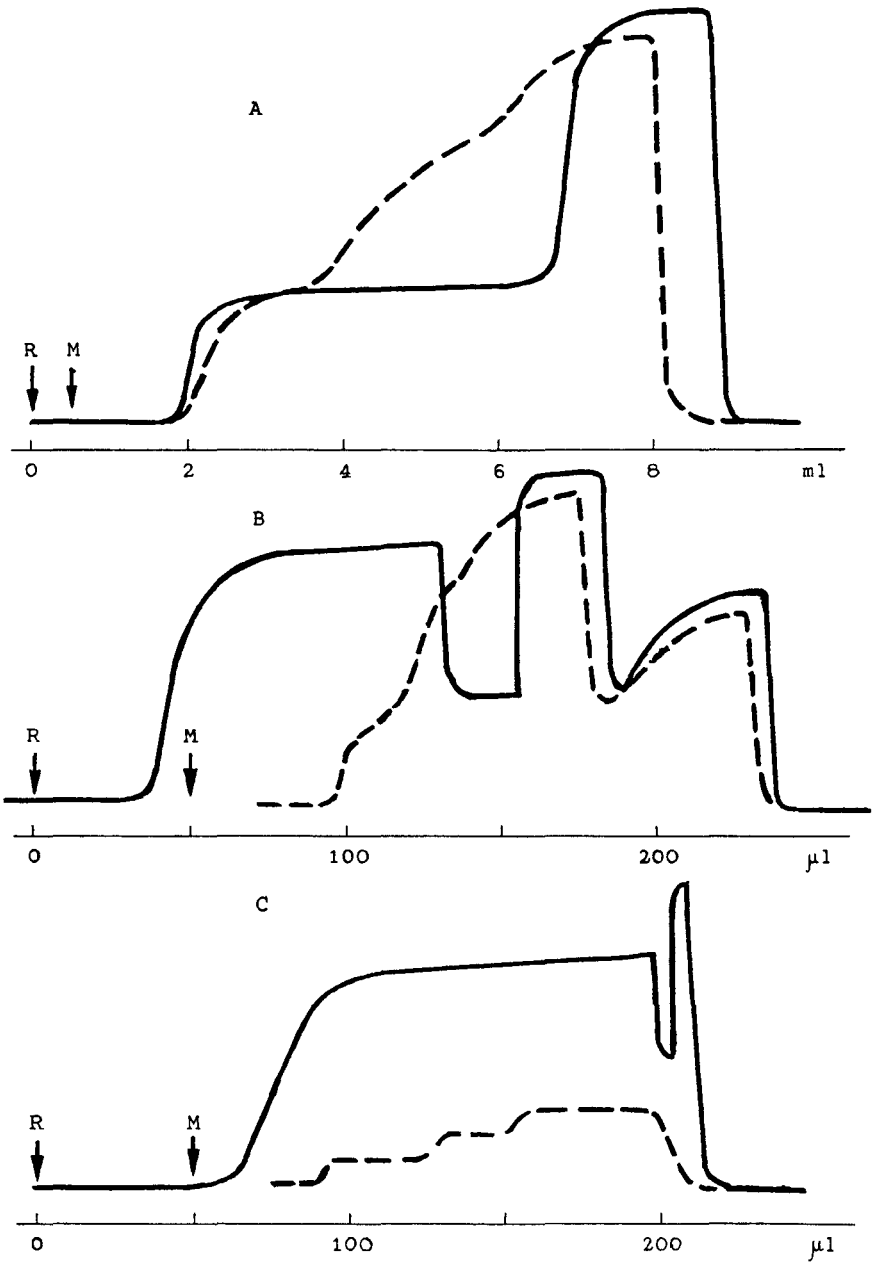

Fig. 10. Restriction (solid lines) and elution (dashed lines) modes of separation on sorbent packing. Columns: (A) 50 × 4.6 I.D., LiChrosorb RP-18, 10 μm; (B,C) 150 × 0.5 mm I.D., Finepack Sil C_{18}, 10 μm. Eluent: water–methanol, (A,B) 3:7, (C) 4:6; UV detection at 260 nm. Restrictor: benzene, (A) 50 μl, (B,C) 4 μl. Mixtures: (A) benzene–toluene, (7:3), 100 μl; (B) chlorobenzene–iodobenzene–*m*-dibromobenzene (10:5:3), 0.7 μl; (C) chlorobenzene–iodobenzene (2:1), saturated solutions in eluent diluted 5-fold, 80 μl.

tions are depicted in Fig. 10. The dashed lines correspond to the ordinary elution mode of separation, which is not satisfactory. When the restrictor is applied (solid lines) the separation is improved, the profile of the curves approximating a rectangular shape. As at non-sorbing support, the restrictor may be either a component of the mixture (Fig. 10A) or not (Fig. 10B).

It is also possible to increase the concentration of a dilute solution up to saturation by means of a restrictor by analogy with displacement enrichment. An example of the simultaneous separation and increase in band concentration of a feed solution diluted in 5-fold compared with the saturated composition is shown in Fig. 10C.

However, there are some differences in the features of the restriction effect on a low-sorbing layer and on a traditional sorbent, especially for strongly sorbed, poorly soluble substances. The latter interact much more weakly with all other components, including the restrictor, than with the solid support. Hence the last zone in Fig. 10B does not change on either chromatogram (the first being obtained with the restrictor and the second without it) whereas the others are altered.

CONCLUSIONS

To perform the chromaextraction method, it is necessary to use a liquid eluent that forms a demixing system with a liquid sample and a non-sorbing column packing that is more wettable by the sample phase than by the eluent phase.

Ordinary isocratic elution of a mixture of substances, characterized by different solubilities in the eluent, allows a partial frontal-type separation to be obtained, so the least soluble component may be obtained at a saturated concentration in the eluent irrespective of the composition of the initial mixture.

To carry out a complete separation an additional component, a restrictor, must be placed on the column previously. As a result, adjacent zones of saturated solutions of individual substances elute in order of decreasing component solubilites. Application of a restrictor also improves separations on sorbent packings when high loadings and limited solubility of the substances occur.

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Effects of molecular structure on parameters *a*, *b* and *c* in the fundamental retention equation

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ABSTRACT

An important aspect of chromatography is the prediction of peak positions. For high-performance liquid chromatography (HPLC), a fundamental retention equation $\ln k' = a + b \ln C_b + cC_b$ has been derived by statistical thermodynamics. The effects of molecular structure on the parameters *a*, *b* and *c* in this equation were investigated theoretically and proved experimentally. The parameter *b* in reversed-phase (RP) HPLC approaches a low constant value, because there is very weak displacement caused by adsorption. However, in normal-phase (NP) HPLC, the displacement by adsorption is strong, and plays an important role in retention. The parameter *b* in NP-HPLC changes slightly when different batches of packing of the same size and with the same mobile phase are used, and the absolute value of this parameter increases slightly and approaches a constant value with increase in carbon number for homologous compounds. The parameter *c* in RP-HPLC is mainly determined by the difference between interactions in solute–strong solvent and solute–weak solvent systems and approaches a constant value even when column systems with the different packings and the same mobile phase are used. The parameter *c* in RP-HPLC can also be quantitatively correlated with structural parameters of the solute such as Van der Waals volume (V_W), dipole moment (μ_A) and hydrogen bond energy (X_{AH}). For non-polar or homologous compounds, a simplified linear relationship between the parameter *c* and V_W can be established. The parameter *c* in NP-HPLC is affected not only by factors valid in RP-HPLC, but also by the displacement caused by adsorption of the solute on the surface. The parameter *a* in both RP- and NP-HPLC follows similar rules to parameter *c*. A linear relationship between k' and amount of C_{18} in RP-HPLC was also found. A good linear relationship between the parameters *a* and *c* occurs only when two of the three structural parameters V_W, μ_A and X_{AH} for solutes are equal or close.

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INTRODUCTION

One of the most important aspects in developing software for expert systems in chromatography is the prediction of peak positions[1,2], which means predicting the capacity factor of a given compound with changes in the operating conditions. For high-performance liquid chromatography (HPLC) the capacity factor and selectivity are changed substantially when the mobile phase composition is changed. Therefore, to solve this problem, we need first to derive an equation that describes the variation of the capacity factor of a given compound when the mobile phase composition is changed over a wide range of concentration, and then to find a means of predicting the values of the parameters in this equation from the molecular structure of the solute.

The capacity factor is determined by the partition coefficient, which is a macro-property of a system. In order to predict macro-properties from the molecular structure, the correct approach is to use statistical thermodynamics (STM)[1,3,4]. There are, of course, other ways of solving this problem, *e.g.*, using classical thermodynamics to obtain the retention equation[5–8] and directly correlating ΔH and ΔS with molecular structure. However, the theoretical correlation between the parameters in the retention equation and molecular structure can only be predicted by STM and statistical quantum chemistry methods[9].

A fundamental retention equation in HPLC has been derived by STM[1,4]. The validity of this equation with changing mobile phase composition over a broad range has been demonstrated in both reversed-phase (RP) HPLC[10–12] and normal-phase (NP) HPLC[13–15]. In this work, a further investigation of effects of molecular structure on the parameters in the fundamental retention equation was carried out. The validity of the theoretical predictions was confirmed experimentally.

FUNDAMENTAL RETENTION EQUATION

The fundamental retention equation valid for high-performance liquid chromatography has been derived as follows[1,4]:

$$\ln k' = a + b \ln C_b + cC_b \tag{1}$$

where

$$a = \ln \frac{N_S}{V_S} - \ln \beta + \frac{\partial N_{B_1}}{\partial N_A} \ln \left[\left(\frac{h^2}{2\pi m_{B_1} kT} \right)^{3/2} \Big/ k_1 V_1 \right] + \frac{3}{2} \ln \frac{h^2}{2\pi m_A kT} +$$

$$+ \frac{1}{kT} \left\{ (K_{AB_0} - K^a_{AB_0}) X_{AB_0} - X^a_A + \frac{\partial N_{B_1}}{\partial N_A} \left[(K_{B_1B_0} - K^a_{B_1B_0}) X_{B_1B_0} - X^a_{B_1} \right] \right\} \tag{2}$$

$$b = \frac{\partial N_{B_1}}{\partial N_A} \left(1 - \frac{1}{n_1} \right) \tag{3}$$

$$c = \frac{1}{kT}\left\{(K_{AB_1} - K^a_{AB_1})X_{AB_1} - (K_{AB_0} - K^a_{AB_0})X_{AB_0} + \right.$$

$$\left. + \frac{\partial N_{B_1}}{\partial N_A}\left[(K_{B_1B_1} - K^a_{B_1B_1})X_{B_1B_1} - (K_{B_1B_0} - K^a_{B_1B_0})X_{B_1B_0}\right]\right\} \quad (4)$$

where A and B_i ($i = 0, 1$) are solute and solvent i (weak and strong solvent), h and k are the Planck and Boltzmann constants, k_1 and n_1 are constants in the Freundlich adsorption isotherm, m_A and m_{B_i} are the molecular weight of solute A and solvent B_i, T is absolute temperature, V_{B_1} is the molar volume of solvent B_1, the X_{AB_1}, X_{AB_0}, X^a_A, $X_{B_1B_1}$, $X_{B_1B_0}$ and $X^a_{B_1}$ are values for interactions of solute–strong solvent, solute–weak solvent, solute–stationary phase, strong solvent–strong solvent, strong solvent–weak solvent and strong solvent–stationary phase, respectively, where the hydrogen bond energy is proportional to the concentration of solvent as assumed if comparing the independence of concentration of solvent in the original papers[1,4] and β is the phase ratio. It can be seen from eqns. 2–4 that the parameter a is mainly determined by the difference between solute–stationary phase and solute–weak solvent interactions as well as solvent–stationary phase interactions, the parameter b is determined by displacement caused by adsorption on the adsorbent surface and the parameter c is mainly determined by the difference between solute–weak solvent and solute–strong solvent interactions as well as solvent–solvent interactions. The validity of eqn. 1 has been proved by describing the variation of the capacity factor by changing the mobile phase composition over a broad range of concentration in both RP-HPLC and NP-HPLC[10–15]. The advantage of this model is that the effects of solute–stationary phase and solute–mobile phase interactions as well as displacement caused by adsorption are simultaneously considered. In a given chromatographic system, the solvent–solvent and solvent–stationary phase interactions are constant, and the solute–stationary phase and solute–solvent B_i interactions can be described by the following equations:

$$X^a_A = -\frac{1}{r^6}\left[\left(\frac{3\alpha_a I_A I_a}{2(I_A + I_a)} + \mu_a^2\right)\alpha_A + \left(\frac{2\mu_a^2}{3kT} + \alpha_a\right)\mu_A^2\right] + Z_a X_{AH} \quad (5)$$

$$X_{AB_i} = -\frac{1}{r^6_{AB_i}}\left[\left(\frac{3\alpha_{B_i} I_A I_B}{2(I_A + I_{B_i})} + \mu_{B_i}^2\right)\alpha_A + \left(\frac{2\mu_{B_i}^2}{3kT} + \alpha_{B_i}\right)\mu_A^2\right] + Z_{AB_i} X_{AH} \quad (6)$$

where I_A, α_A, μ_A, I_a, α_a, μ_a and I_{B_i}, α_{B_i}, μ_{B_i} are the approximate ionization energy, polarizability and dipole moment of the solute, stationary phase and solvent i, respectively, X_{AH} the contribution of the hydrogen bond energy of the solute, Z_a and Z_{AB_i}, are parameters related to the behaviour of the stationary phase and solvent i and r and r_{AB_i} are the interaction distance of solute–stationary phase and solute–solvent i, respectively, and to a first approximation are assumed to be constant for different compounds.

FACTORS AFFECTING THE PARAMETER b

It can be seen from eqn. 3 that the parameter b is determined by n_1 and $\partial N_{B_1}/\partial N_A$, where n_1 is a parameter of the Freundlich adsorption isotherm ($N^a_{B_1} = k_1 C b^{1/n_1}$) on a stationary surface for a strong solvent and relates to the behaviours of the adsorbent surface and a strong solvent, and $\partial N_{B_1}/\partial N_A$ indicates how many strong solvent molecules are displaced by the adsorption of one solute molecule. For a given system, the strength of the solute–adsorbent interaction and the solute molecular size are the factors that determine $\partial N_{B_1}/\partial N_A$. The stronger the solute–adsorbent interaction and the larger the molecular size of the solute, the more strong solvent molecules can be displaced by adsorption of one molecule of the solute on the adsorbent surface. In RP-HPLC a polar solvent molecule is less easily adsorbed on a C_{18} surface than a polar solvent molecule on silica in the NP-HPLC, so that $\partial N_{B_1}/\partial N_A$ approaches a small constant value. Table I lists the b values observed in RP-HPLC, which agree with the theoretical values.

The value of C_b in the most RP-HPLC separations is higher than 0.1 volume fraction of the strong solvent, but in most NP-HPLC separations C_b is less than 0.1 or even 0.001 volume fraction of the strong solvent. The absolute value of ln C_b in RP-HPLC is much smaller than that in NP-HPLC in most separations, so the contribution of term b ln C_b in eqn. 1 to the retention value in RP-HPLC can be neglected in the case of small concentrations of the strong solvent. However, in NP-HPLC the solute–adsorbent interaction, which is mainly due to interactions between polar groups of the solute and silanol groups, is strong and the absolute value of ln C_b is large, so the contribution of b ln C_b to the retention value in NP-HPLC is significant. For a homologous series, the parameter b is mainly determined by the interaction between polar groups and silanol groups on the surface, and the absolute value of b should increase slightly with increasing number of methylene groups in the solute molecule and approach a constant value at a certain number of carbon atoms. The validity of this conclusion is supported by the experimental data given in Table II for the homologous series of benzoates of n-alkanols.

TABLE I

PARAMETER b OBSERVED IN RP-HPLC WITH DIFFERENT C_{18} PACKINGS

Solute	*YQG*[a]	*ES*[b]	*Nucleosil*	*LiChrosorb*	*B4*[c]	*B8*[c]
Benzene	0	−0.001	−0.003	−0.001	−0.003	−0.001
Naphthalene	−0.01	−0.003	−0.007	−0.001	−0.003	−0.003
Biphenyl	−0.01	−0.005	−0.007	−0.002	−0.004	−0.001
Fluorene	−0.005	−0.007	−0.003	−0.003	−0.002	0
Phenanthrene	−0.003	−0.005	−0.005	–	–	–
Anthracene	−0.003	−0.007	−0.005	–	–	–
Chrysene	−0.004	−0.003	−0.002	–	–	–
Toluene	−0.005	−0.002	−0.005	−0.006	−0.003	0
Ethylbenzene	−0.004	−0.003	−0.005	−0.01	–	–

[a] YQG-C_{18} is made by Tinjing Chemical Reagent Factory (China).
[b] ES-C_{18} is made by ES Industry Inc.
[c] B4 and B8 are homemade silica-based C_{18} packing materials.

TABLE II

PARAMETER b OBSERVED IN THE ADSORPTION CHROMATOGRAPHY OF BENZOATES OF HOMOLOGOUS n-ALKANOLS IN THE SYSTEM OF YWG–SILICA GEL (5 μm) PACKING AND HEXANE–ETHYL ACETATE MOBILE PHASE (ETHYL ACETATE FROM 0.1 TO 3.0%, v/v)

Data recalculated from ref. 16.

n_c	b	n_c	b
1	−0.64	5	−0.75
2	−0.69	6	−0.74
3	−0.72	7	−0.77
4	−0.74	8	−0.77

TABLE III

PARAMETER b FOR EIGHT POLAR COMPOUNDS OBSERVED IN A COLUMN SYSTEM WITH DIFFERENT BATCHES OF YWG–SILICA GEL (5 μm) PACKING AND HEXANE–ETHYL ETHER MOBILE PHASE

Compound	*Parameter*[a]		
	b'	b''	b'''
2-Methylnitrobenzene	−0.41	−0.42	−0.50
Nitrobenzene	−0.37	−0.37	−0.40
Ethoxybenzene	−0.54	−0.50	–
4-Chloronitrobenzene	−0.49	−0.50	−0.51
2-Chloronitrobenzene	−0.27	−0.26	−0.32
n-Butyl benzoate	−0.78	−0.82	–
n-Pentyl benzoate	−0.78	−0.81	–
Ethyl benzoate	−0.74	−0.79	–

[a] b' recalculated from ref. 17; mobile phase composition: ethyl ether from 2.0 to 70% (v/v); b'' recalculated from ref. 14; mobile phase composition: ethyl ether from 0.1 to 60% (v/v); b''' recalculated from ref. 15; mobile phase composition: ethyl ether from 0.2 to 60% (v/v).

The parameter b changes very little when different batches of packing material with the same grain size and the same mobile phase are used, because the behaviour of the adsorbing surface is very similar. Table III gives the values of the parameter b observed in adsorption chromatography, which are in good agreement with the theoretical values.

FACTORS AFFECTING PARAMETER c

The parameter c in RP-HPLC is mainly determined by the difference between solute–strong solvent and solute–weak solvent interactions, and is almost independent of the behaviour of the solute adsorbed on the surface. It is a constant when different packings and the same mobile phase are used. Table IV gives the values of the parameter c observed with different C_{18} packings for various solutes.

On the other hand, the polarizability of a solute is proportional to its Van der Waals volume:

$$\alpha_A = e + fV_w \tag{7}$$

TABLE IV

PARAMETER c OBSERVED WITH DIFFERENT C_{18} PACKINGS WITH WATER–METHANOL MOBILE PHASE (METHANOL FROM 60 TO 95%, v/v) FOR VARIOUS COMPOUNDS

Solute	*YQG*	*ES*	*Nucleosil*	*LiChrosorb*	*B4*	*B8*
Benzene	−2.69	−2.62	−2.71	−2.80	−2.86	−2.75
Naphthalene	−3.50	−3.49	−3.62	−3.59	−3.78	−3.67
Biphenyl	−4.14	−4.10	−4.26	−3.83	−4.40	−4.27
Phenanthrene	−4.54	−4.53	−4.30	−4.35	−4.56	−4.46
Anthracene	−4.44	−4.50	−4.41	–	–	–
Chrysene	−5.09	−5.04	−5.18	–	–	–
1,4-Diphenylbenzene	−5.59	−5.55	−5.57	–	–	–
Anisole	−2.70	−2.63	−2.74	−2.82	−2.80	−2.71
Benzyl alcohol	−2.05	−1.93	−2.00	−2.12	–	–
Acetophenone	−2.30	−2.18	−2.30	–	–	–
p-Nitrotoluene	−3.00	−2.97	−3.05	–	–	–
n-Butyl benzoate	−4.05	−4.00	−4.10	–	–	–

On substitution of eqns. 5 and 6 together with eqn. 7 into eqn. 4, the parameter c can be expressed as:

$$c = k_2 + k_3 V_w + k_4 \mu_A^2 + k_5 X_{AH} \tag{8}$$

where

$$k_2 = \frac{e}{kT}\left[(K_{AB_0} - K^a_{AB_0})\frac{1}{r^6_{AB}}\left(\frac{3\alpha_{B_0} I_A I_{B_0}}{2(I_A + I_{B_0})} + \alpha_{B_0}\right) - (K_{AB_1} - K^a_{AB_1})\frac{1}{r^6_{AB_1}}\left(\frac{3\alpha_{B_1} I_A I_{B_1}}{2(I_A + I_{B_1})} + \alpha_{B_1}\right)\right] \tag{9}$$

$$k_3 = \frac{f}{kT}\left[(K_{AB_0} - K^a_{AB_0})\frac{1}{r^6_{AB_0}}\left(\frac{3\alpha_{B_0} I_A I_{B_0}}{2(I_A + I_{B_0})} + \alpha_{B_0}\right) - (K_{AB_1} - K^a_{AB_1})\frac{1}{r^6_{AB_1}}\left(\frac{3\alpha_{B_0} I_A I_{B_0}}{2(I_A + I_{B_0})} + \alpha_{B_0}\right)\right] \tag{10}$$

$$k_4 = \frac{1}{kT}\left[(K_{AB_0} - K^a_{AB_0})\frac{1}{r^6_{AB_0}}\left(\frac{\mu^2_{B_0}}{3kT} + \alpha_{B_0}\right) - (K_{AB_1} - K^a_{AB_1})\cdot\frac{1}{r^6_{AB_1}}\left(\frac{\mu^2_{B_1}}{3kT} + \alpha_{B_1}\right)\right] \tag{11}$$

$$k_5 = \frac{1}{kT}[(K_{AB_1} - K^a_{AB_1})Z_{AB_1} - (K_{AB_0} - K^a_{AB_0})Z_{AB_0}] \tag{12}$$

It can be seen from eqn. 8 that the parameter c is determined by the Van der Waals volume, dipole moment and hydrogen bond energy of the solute in a given mobile phase system. V_w can be calculated by Bondi's method[18], dipole moment data are available for some solutes, but data on hydrogen bond energies are scarce[19]. In fact, the hydrogen bond energy between molecules is also affected by the sourrounding functional groups. For example, if the parent compound to which a hydroxy group is attached is a hydrocarbon, then the hydrogen bond energy between molecules can be 0.5–1.0 kcal/mol higher than that between water molecules[20]. On the other hand, the possibility of hydrogen donors and/or acceptors for the solute and solvent in chromatography must be considered simultaneously[19].

Tables V and VI show the values of V_w, μ_A and X_{AH} for some solutes and a comparison of calculated and experimental values of parameter c. The results in Tables V and VI show the good correlation between the parameter c and structural parameters of the solute.

If a solute is non-polar, then $\mu_A = 0$ and $X_{AH} = 0$ and there should be a linear relationship between the parameter c and Van der Waals volume of the solute (V_w).

TABLE V

VALUES OF V_w, μ_A AND X_{AH} FOR VARIOUS SOLUTES AND COMPARISON OF CALCULATED AND EXPERIMENTAL c VALUES WITH METHANOL–WATER MOBILE PHASE (METHANOL FROM 40 TO 70%, v/v)

Data are recalculated from ref. 21.

Solute	V_w	μ_A	X_{AH}	c' (exp.)[a]	c' (calc.)[a,b]	Δ
Benzene	43.36	0	0	−2.95	−2.67	0.28
Chlorobenzene	57.48	1.58	0	−3.75	−3.45	0.39
Toluene	59.51	0.43	0	−3.56	−3.56	0
Bromobenzene	60.96	1.53	0	−3.93	−3.64	0.29
Iodobenzene	65.48	1.28	0	−4.13	−3.89	0.24
Ethylbenzene	69.74	0.35	0	−4.06	−4.13	−0.07
o-Xylene	70.66	0	0	−3.87	−4.18	−0.31
Aniline	56.38	1.56	15	−2.38	−2.28	0.10
Phenol	53.88	1.55	12	−2.66	−2.36	0.30
Methoxybenzene	63.21	1.25	6	−3.16	−3.32	−0.16
Benzaldehyde	60.06	2.80	10	−2.62	−2.63	−0.01
Benzyl alcohol	64.11	1.67	16	−2.79	−2.84	−0.05
Nitrobenzene	62.64	3.93	8	−3.03	−3.12	−0.09
Benzonitrile	60.54	3.93	8	−3.05	−3.00	0.05
Benzyl cyanide	70.77	3.50	12	−3.27	−3.27	0
1-Chloro-4-nitrobenzene	72.12	2.60	9	−3.45	−3.58	−0.13
1-Bromo-4-nitrobenzene	75.23	1.94	9	−3.63	−3.76	−0.13
4-Nitrophenol	68.14	4.76	16	−2.83	−2.82	0.01
1,4-Dimethoxybenzene	77.06	1.70	16	−3.27	−3.34	−0.07
2,4-Dinitrobenzene	88.07	4.33	20	−3.33	−3.62	−0.29
4-Methoxybenzaldehyde	74.41	3.26	16	−3.17	−3.18	−0.01
4-Hydroxybenzaldehyde	65.58	4.22	20	−2.69	−2.39	0.30

[a] $c' = c/\ln 10$.

[b] $c' = -0.2887 + 0.07414\ X_{AH} - 0.05502\ V_w - 0.001535\ \mu_A^2$; S.D. = 0.2021, average error = 0.1535, $N = 22$.

TABLE VI

VALUES OF V_w, μ_A AND X_{AH} FOR PHENOLS AND CARBOXYLIC ACIDS AND COMPARISON OF CALCULATED AND EXPERIMENTAL c VALUES WITH ACETONITRILE–WATER MOBILE PHASE (ACETONITRILE FROM 20 TO 40%, v/v)

Data are recalculated from ref. 22.

Solute	V_w	μ_A	X_{AH}	c' (exp.)[a]	c' (calc.)[a,b]	Δ
2-Methylphenol	65.03	1.41	6.5	−3.11	−3.11	0
3-Methylphenol	65.03	1.54	6.5	−3.17	−3.12	−0.05
4-Methylphenol	65.03	1.54	6.5	−3.17	−3.12	−0.03
2,3-Dimethylphenol	76.18	1.25	7.0	−3.68	−3.76	−0.08
2,4-Dimethylphenol	76.18	1.39	7.0	−3.76	−3.77	−0.01
2,5-Dimethylphenol	76.18	1.44	7.0	−3.73	−3.78	−0.05
3,4-Dimethylphenol	76.18	1.53	7.0	−3.73	−3.79	−0.06
3,5-Dimethylphenol	76.18	1.76	7.0	−3.83	−3.82	0.01
Benzoic acid	63.38	1.72	7.5	−3.34	−3.31	0.03
3-Methylbenzoic acid	76.51	2.75	7.5	−4.09	−4.15	−0.06
2-Methylbenzoic acid	76.51	1.70	7.5	−3.82	−3.95	−0.13
2-Chlorobenzoic acid	74.84	2.49	7.5	−3.67	−4.01	−0.34
2-Bromobenzoic acid	77.96	1.91	7.5	−3.77	−4.06	−0.29
3-Bromobenzoic acid	77.96	2.17	7.5	−4.51	−4.19	0.32
4-Bromobenzoic acid	77.96	2.10	7.5	−4.59	−4.10	0.41
trans-Cinnamic acid	82.32	2.26	7.5	−4.33	−4.33	0

[a] $c' = c/\ln 10$.

[b] $c' = 1.7794 - 0.2490\,X_{AH} - 0.04895\,V_w - 0.04216\,\mu_A^2$; S.D. = 0.2026, average error = 0.1294, $N = 16$.

Fig. 1 shows the relationship between c and V_w observed in an RP-HPLC experiment.

If solutes belong to a homologous series, the same dipole moments and hydrogen bond energies are assumed and Van der Waals volume can be described as:

$$V_w = \sum_i n_i \Delta V_{w(i)} + n_c \Delta V_{w(CH_2)} \tag{13}$$

where n_i and $\Delta V_{w(i)}$ are the numbers of group i and the Van der Waals volume contributed by group i and n_c and $\Delta V_{w(CH_2)}$ are the numbers of methylene groups and the Van der Waals volume contributed by a methylene group. Substitution of eqn. 13 together with μ_A and X_{AH} in eqn. 8 gives the parameter c for homologous compounds:

$$c = k_6 + k_7 n_c \tag{14}$$

where

$$k_6 = k_2 + k_4\mu_A^2 + k_5 X_{AH} + k_3\left[\sum_i n_i \Delta V_{w(i)}\right] \tag{15}$$

$$k_7 = k_3\,[\Delta V_{w(CH_2)}] \tag{16}$$

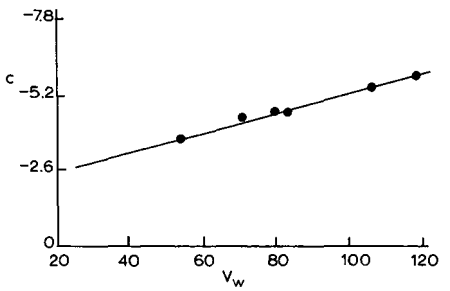


Fig. 1. Linear relationship between c and V_w for some polynuclear aromatic hydrocarbons observed in an RP-HPLC column system with Nucleosil-ODS packing and water–methanol mobile phase (methanol from 60 to 95%, v/v).

Eqns. 14–16 mean that there is a linear relationship between c and carbon number for homologous compounds, as shown by the data in Table VII.

In NP-HPLC, $\partial N_{B_1}/\partial N_A$ is large and the effect of displacement of a solute by adsorption on the parameter c cannot be neglected. For homologous compounds in adsorption chromatography, the relationship between carbon number and the parameter c shown in eqn. 14 must be corrected for the contribution of adsorption of the solute:

$$c = k_6 + k_7 n_c + mb \tag{17}$$

where

$$m = \frac{1}{kT\left(1 - \frac{1}{n_1}\right)} [(K_{B_1B_1} - K^a_{B_1B_1})X_{B_1B_1} - (K_{B_1B_0} - K^a_{B_1B_0})X_{B_1B_0}]$$

TABLE VII

LINEAR RELATIONSHIPS BETWEEN c AND CARBON NUMBER FOR HOMOLOGOUS SERIES

Data recalculated from ref. 23. Homologues: A, 3,5-dinitrobenzoates of *n*-alkanols; B, *p*-bromophenacyl esters of lower saturated carboxylic acids; C, 1,2-naphthoylbenzimidazole 6-sulphonamides of *n*-alkylamines. Binary mobile phases: M_1 = methanol–water (methanol from 70 to 90%, v/v); M_2 = dioxane–water (dioxane from 50 to 75%, v/v): M_3 = acetonitrile–water (acetonitrile from 60 to 80%, v/v).

Mobile phase	*Homologous series*								
	A			*B*			*C*		
	k_6	k_7	r^a	k_6	k_7	r^a	k_6	k_7	r^a
M_1	−6.50	−1.01	0.9984	−6.49	−0.944	0.9955	−6.59	−0.999	0.9984
M_2	−6.89	−1.30	0.9963	−6.13	−1.29	0.9991	−8.36	−1.06	0.9938
M_3	−6.62	−0.327	0.9924	−5.79	−0.398	0.9868	−2.97	−0.529	0.9780

[a] Regression coefficient.

TABLE VIII

COMPARISON OF EXPERIMENTAL AND CALCULATED c VALUES FOR HOMOLOGOUS BENZOATES OF n-ALKANOLS

Data from ref. 16.

Parameter	n_c			
	1	*2*	*3*	*4*
c (exp.)	−0.39	−0.45	−0.58	−0.83
c (calc.)	−0.39	−0.43	−0.60	−0.83

and k_6 and k_7 are as in eqns. 15 and 16. Table VIII gives the results for the comparison of experimental measured parameters c with those calculated by eqn. 17 for a homologous series of benzoates of n-alkanols. The results in Table VIII describe well the variation of the parameter c for homologous derivatives separated by NP-HPLC.

FACTORS AFFECTING THE PARAMETER a

The parameter a is influenced by the behaviours of the stationary phase, solvent and solute. In a given reversed-phase system, the parameter a can also be correlated with structural parameters of the solute and can be expressed as:

$$a = k'_2 + k'_3 V_w + k'_4 \mu_A^2 + k'_5 X_{AH} \tag{18}$$

where

$$k'_2 = \ln\left(\frac{N_S}{V_S}\right) - \ln\beta + \frac{3}{2}\ln\left(\frac{h^2}{2\pi m_A kT}\right) + \\ + \frac{e}{kT}\left\{(K^a_{AB} - K_{AB_0})\frac{1}{r^6_{AB_0}}\left[\frac{3\alpha_{B_0} I_A I_{B_0}}{2(I_A + I_{B_0})} + \alpha_{B_0}\right] - \frac{1}{r^6}\left[\frac{3\alpha_a I_a I_A}{2(I_A + I_a)} + \alpha_a\right]\right\} \tag{19}$$

$$k'_3 = \frac{f}{kT}\left\{\frac{1}{r^6}\left[\frac{3\alpha_a I_A I_a}{2(I_A + I_a)} + \alpha_a\right] - (K_{AB_0} - K^a_{AB_0})\frac{1}{r^6_{AB_0}}\left[\frac{3\alpha_{B_0} I_A I_{B_0}}{2(I_A + I_{B_0})} + \alpha_{B_0}\right]\right\} \tag{20}$$

$$k'_4 = \frac{1}{kT}\left[\left(\frac{2\mu_A^2}{3kT} + \alpha_a\right) - (K_{AB_0} - K^a_{AB_0})\left(\frac{2\mu^2_{B_0}}{3kT} + \alpha_{B_0}\right)\right] \tag{21}$$

$$k'_5 = \frac{1}{kT}[Z_a - (K_{AB_0} - K^a_{AB_0})Z_{AB_0}] \tag{22}$$

It is possible to correlate the parameter a quantitatively with the structural parameters V_w, μ_A and X_{AH} of a solute as discussed above. Table IX illustrates the quantitative correlation between parameter a and V_w, μ_A and X_{AH} observed in RP-HPLC with methanol–water for 22 compounds and acetonitrile–water for 16 compounds, and demonstrates the validity of the theoretical deduction.

TABLE IX

COMPARISON OF EXPERIMENTAL AND CALCULATED a VALUES

Values of V_w, μ_A and X_{AH} for solutes and mobile phase compositions as in Tables V and VI.

Methanol–water				*Acetonitrile–water*			
Solute	a' *(exp.)*[a]	a' *(calc.)*[a,b]	Δ	*Solute*	a' *(exp.)*[a]	a' *(calc.)*[a,c]	Δ
Benzene	2.45	2.53	0.08	2-Methylphenol	1.67	1.68	0.01
Chlorobenzene	3.22	2.97	−0.25	3-Methylphenol	1.65	1.67	0.02
Toluene	3.12	3.12	0	4-Methylphenol	1.65	1.67	0.02
Bromobenzene	3.42	3.16	−0.26	2,3-Dimethylphenol	2.10	2.09	−0.01
Iodobenzene	3.68	3.42	−0.26	2,4-Dimethylphenol	2.14	2.09	−0.05
Ethylbenzene	3.68	3.67	−0.01	2,5-Dimethylphenol	2.14	2.09	−0.05
o-Xylene	3.60	3.72	0.12	3,4-Dimethylphenol	2.12	2.07	−0.05
Aniline	1.36	1.43	0.07	3,5-Dimethylphenol	2.04	2.08	0.04
Phenol	1.56	1.59	0.03	Benzoic acid	1.47	1.42	−0.05
Methoxybenzene	2.55	2.70	0.15	3-Methylbenzoic acid	2.00	1.95	−0.05
Benzaldehyde	1.92	2.04	0.12	2-Methylbenzoic acid	1.87	2.01	0.14
Benzyl alcohol	1.57	1.74	0.17	2-Chlorobenzoic acid	1.69	1.89	0.20
Nitrobenzene	2.06	2.23	0.17	2-Bromobenzoic acid	1.80	2.06	0.26
Benzonitrile	2.06	2.12	0.06	3-Bromobenzoic acid	2.33	2.05	−0.28
Benzyl cyanide	2.15	2.67	0.52	4-Bromobenzoic acid	2.38	2.05	−0.33
1-Chloro-4-nitrobenzene	2.82	2.80	−0.02	*trans*-Cinnamic acid	2.08	2.24	0.16
1-Bromo-4-nitrobenzene	3.00	3.01	0.01				
4-Nitrophenol	1.77	1.61	−0.16				
1,4-Dimethoxybenzene	2.55	2.44	−0.11				
2,4-Dinitrobenzene	2.62	2.36	−0.26				
4-Methoxybenzaldehyde	2.23	2.32	0.09				
4-Hydroxybenzaldehyde	1.36	1.16	−0.20				

[a] $a' = a/\ln 10$.

[b] $a' = -0.07132 + 0.05372\,V_w - 0.01747\,\mu_A^2 - 0.09885\,X_{AH}$; S.D. = 0.1812, average error = 0.1400, $N = 22$.

[c] $a' = -0.1094 + 0.04441\,V_w - 0.01305\,\mu_A^2 + 0.07414\,X_{AH}$; S.D. = 0.1821, average error = 0.1389, $N = 16$.

If the solutes are non-polar, then $\mu_A = 0$ and $X_{AH} = 0$ and eqn. 18 can be simplified to:

$$a = k'_2 + k'_3 V_w \tag{23}$$

Eqn. 23 means that there should be a linear relationship between the parameter a and V_w for non-polar compounds. The validity is confirmed by the results shown in Fig. 2.

If the solutes belong to a homologous series, there should be a linear relationship between the parameter a and the carbon numbers of the homologous substances:

$$a = k'_6 + k'_7 n_c \tag{24}$$

where

$$k'_6 = k'_2 + k'_3 \left[\sum_i n_i \Delta V_{w(i)} \right] + k'_4 \mu_A^2 + k'_5 X_{AH} \tag{25}$$

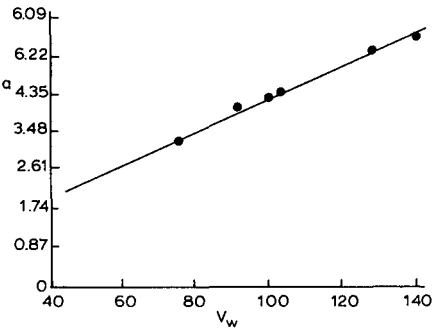


Fig. 2. Linear relationship between a and V_w for some non-polar compounds observed with the same system as in Fig. 1.

$$k'_7 = k'_3 [\Delta V_{w(CH_2)}] \tag{26}$$

Table X gives the results of the linear regression of parameter a and carbon numbers of the homologous series, showing consistancy with theory.

In adsorption chromatography, the effect of displacement by adsorption on the parameter a must be considered for a homologous series. Then the parameter a can be expressed as:

$$a = k'_6 + k'_7 n_c + m'b \tag{27}$$

where

$$m' = \frac{1}{kT\left(1 - \frac{1}{n_1}\right)} [(K_{B_1B_0} - K^a_{B_1B_0})X_{B_1B_0} - X^a_{B_1}]$$

and k'_6 and k'_7 are as in eqns. 25 and 26. Eqn. 27 derived theoretically describes well the dependence of the parameter a on the carbon numbers n_c and the parameter b (Table XI).

TABLE X

LINEAR RELATIONSHIP BETWEEN a AND CARBON NUMBERS FOR HOMOLOGOUS SERIES

Homologues A–C and mobile phases M_1–M_3 as in Table VII. Data recalculated from ref. 23.

Mobile phase	*Homologous series*								
	A			*B*			*C*		
	k'_6	k'_7	r^a	k'_6	k'_7	r^a	k'_6	k'_7	r^a
M_1	4.04	1.18	0.9991	4.00	1.10	0.9970	4.89	1.10	0.9994
M_2	3.93	1.26	0.9990	3.27	1.23	0.9998	3.80	1.05	0.9971
M_3	3.51	0.592	0.9991	3.09	0.628	0.9967	1.59	0.677	0.9883

[a] Regression coefficient.

TABLE XI

COMPARISON OF EXPERIMENTAL AND CALCULATED (EQN. 27) a VALUES FOR HOMOLOGOUS BENZOATES OF n-ALKANOLS

$k'_6 = -2.22$, $k'_7 = -0.161$, $m' = -0.105$. Data from ref. 16.

n_c	a (exp.)	a (calc.)	Δ
1	−2.14	−2.31	−0.17
2	−2.50	−2.47	0.03
3	−2.76	−2.63	0.13
4	−2.95	−2.94	0.01
5	−3.03	−2.95	0.08
6	−3.13	−3.11	0.02
7	−3.31	−3.26	0.04
8	−3.28	−3.42	−0.14

Another problem is to establish how the amount of bonded hydrocarbon chain (N_S) covering the stationary phase affects the retention value in RP-HPLC. From eqns. 1–4, it can be seen that the effect of N_S on the retention is given by the change in the parameter a when the other operating conditions remain constant:

$$a = \ln N_S + C \tag{28}$$

where

$$C = -\ln V_S - \ln \beta + \frac{\partial N_{B_1}}{\partial N_A} \ln \left[\left(\frac{h^2}{2\pi m_{B_1} kT} \right)^{3/2} \Big/ k_1 V_1 \right] + \frac{3}{2} \ln \left(\frac{h^2}{2\pi m_A kT} \right) +$$

$$+ \frac{1}{kT} \left\{ (K_{AB_0} - K^a_{AB_0}) X_{AB_0} - X^a_A + \frac{\partial N_{B_1}}{\partial N_A} [(K_{B_1B_0} - K^a_{B_1B_0}) X_{B_1B_0} - X^a_{B_1}] \right\} \tag{29}$$

By substituting eqn. 28 together with eqns. 2–4 into eqn. 1 and rearrangement, we obtain the following expression:

$$k' = SN_S \tag{30}$$

where

$$S = \exp (b \ln C_b + cC_b + C) \tag{31}$$

Eqn. 31 means that there is a linear relationship between k' and N_S which passes through the origin (Figs. 3 and 4).

RELATIONSHIP BETWEEN PARAMETERS a AND c

It has been reported that a linear relationship between the parameters a and c was observed for some compounds[7,23]. From the results discussed in the previous two

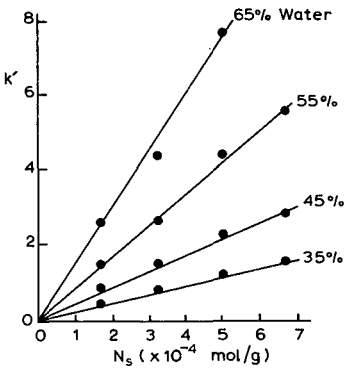


Fig. 3. Linear relationship between k' and N_S for nitrobenzene at different compositions of methanol–water mobile phase. Data replotted from ref. 24.

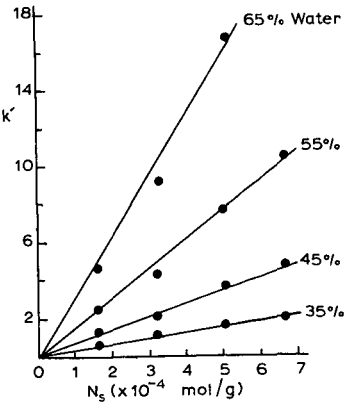


Fig. 4. Linear relationship between k' and N_S for methyl benzoate at different compositions of methanol–water mobile phase. Data replotted from ref. 24.

sections, it can be concluded that for a given RP-HPLC system it is possible to obtain a good linear relationship between parameters a and c if two of the three structural parameters V_w, μ_A and X_{AH} are equal or close. For non-polar compounds and homologous series of compounds this relationship can be expressed by the following equations:

$$\text{non-polar compounds: } a = k_8 + k_9 c \tag{32}$$

$$\text{homologous series: } a = k'_8 + k'_9 c \tag{33}$$

where

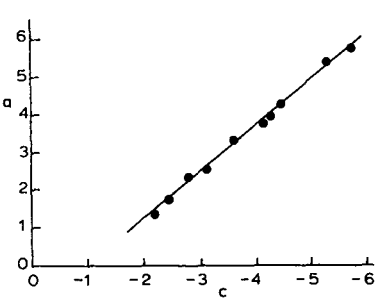
$$k_8 = k'_2 - k'_3 k_2/k_3; \qquad k_9 = k'_3/k_3 \tag{34}$$

$$k'_8 = k'_6 - k'_7 k_6/k_7; \qquad k'_9 = k'_7/k_7 \tag{35}$$

It can be seen from eqns. 34 and 35 that the slope of the linear relationship between a and c is mainly determined by the ratio of the difference between the solute–stationary phase and solute–weak solvent dispersive interactions to the difference between the solute–strong solvent and solute–weak solvent dispersive interactions, the intercept being related to the various physico-chemical behaviours of the stationary phase, solute and solvents. Figs. 5 and 6 show the linear relationship between a and c for non-polar compounds and homologous series, respectively. Table XII gives the results of a comparison of k'_8 and k'_9 calculated by eqn. 35 and those obtained by regression from a and c directly for homologous series. These results for non-polar and homologous compounds confirm the theoretical data.

It must be pointed out that k'_9 for homologues in a dioxane–water binary mobile phase is less than -1, which means that

$$-k'_7 \geqslant k_7 \tag{36}$$



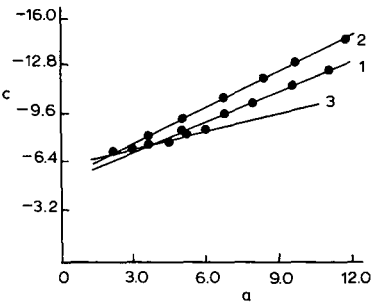


Fig. 5. Linear relationship between a and c for ten non-polar compounds observed with the same column system as in Fig. 1.

Fig. 6. Linear relationship between a and c for homologous 3,5-dinitrobenzoates of n-alkanols in different binary mobile phases: 1 = methanol–water; 2 = acetonitrile–water; 3 = dioxane–water (see Table VII). Data replotted from ref. 22.

After substitution of eqns. 16 and 27 into eqn. 36 and rearrangement, we obtain:

$$\frac{1}{r^6}\left(\frac{3\alpha_a I_A I_a}{2(I_A + I_a)} + \mu_a^2\right) \leqslant \frac{1}{r_{AB_1}^6}(K_{AB_1} - K_{AB_1}^a)\left(\frac{3\alpha_{B_1} I_A I_{B_1}}{2(I_A + I_{B_1})} + \mu_{B_1}^2\right) \quad (37)$$

Eqn. 37 means that the dispersive interaction between the solute and the stationary

TABLE XII

COMPARISON OF EXPERIMENTAL AND CALCULATED k'_8 AND k'_9 VALUES (EQN. 32) FOR HOMOLOGOUS SERIES

Data recalculated from ref. 23. Homologues A–C and mobile phases M_1–M_3 as in Table IV.

Mobile phase	*Homologous series*								
	A			*B*			*C*		
	k'_9 *(exp.)*	k'_9 *(calc.)*	Δ *(%)*[a]	k'_9 *(exp.)*	k'_9 *(calc.)*	Δ *(%)*[a]	k'_9 *(exp.)*	k'_9 *(calc.)*	Δ *(%)*[a]
M_1	−0.859	−0.856	0.07	−0.859	−0.855	0.47	−0.908	−0.911	−0.27
M_2	−1.037	−1.035	0.24	−1.050	−1.049	0.11	−1.011	−1.009	0.24
M_3	−0.558	−0.554	0.71	−0.643	−0.634	1.34	−0.802	−0.780	2.78
	k'_8 *(exp.)*	k'_8 *(calc.)*	Δ *(%)*	k'_8 *(exp.)*	k'_8 *(calc.)*	Δ *(%)*	k'_8 *(exp.)*	k'_8 *(calc.)*	Δ *(%)*
M_1	−3.039	−3.046	−0.20	−3.048	−3.031	−0.07	−2.144	−2.135	0.40
M_2	−2.807	−2.828	−0.75	−2.702	−2.710	−0.31	−4.50	−4.526	−0.58
M_3	−4.651	−4.675	−0.42	−3.827	−3.779	1.27	−1.692	−1.737	−2.68

[a] Relative error: $\Delta\ (\%) = \dfrac{k'_{i\,(exp.)} - k'_{i\,(calc.)}}{k'_{i\,(exp.)}} \cdot 100.$

phase is weaker than that between the solute and a strong solvent. If a methanol–water or acetonitrile–water binary mobile phase is used, then $k'_q \geqslant -1$, which means that:

$$\frac{1}{r^6}\left[\frac{3\alpha_a I_A I_a}{2(I_A + I_a)} + \mu_a^2\right] \geqslant \frac{1}{r_{AB_1}^6}(K_{AB_1} - K_{AB_1}^a)\left[\frac{3\alpha_{B_1} I_A I_{B_1}}{2(I_A + I_{B_1})} + \mu_{B_1}^2\right] \quad (38)$$

Eqn. 38 shows that the dispersive interaction between the solute and a strong solvent is weaker than that between the solute and the stationary phase. These results confirm that the dispersive interaction of dioxane is stronger than that of methanol and acetonitrile and the stationary phase affects the retention and selectivity in both NP- and RP-HPLC.

CONCLUSION

The parameter b in RP-HPLC approaches to a small constant value because the displacement by adsorption is very weak. In NP-HPLC, this displacement is strong and the parameter b changes slightly when different batches of packing of the same size and the same mobile phase are used; the absolute value of the parameter b increases slightly and approaches a constant value with increasing carbon numbers for homologous series. The parameter c in RP-HPLC is mainly determined by the difference between solute–strong solvent and solute–weak solvent interactions and approaches a constant value when different packings and the same mobile phase are used. The parameter c in RP-HPLC can be correlated with structural parameters of the solute such as Van der Waals volume (V_w), dipole moment (μ_A) and hydrogen bond energy (X_{AH}). For compounds such as non-polar or homologous series, a simplified linear relationship between the parameters c and V_w can be established. The parameter c in NP-HPLC is also influenced by displacement of the solute and the relationships discussed for parameter c in RP-HPLC must be modified to take account of this effect. The parameter a in both RP- and NP-HPLC follows similar relationships to the parameter c. A linear relationship between k' and coverage of C_{18} in RP-HPLC has been derived theoretically and confirmed experimentally. There is a good linear relationship between the parameters a and c only when two of the three structural parameters V_w, μ_A and X_{AH} of solutes are equal or close and the effect of displacement of solute by adsorption on the parameters a and c can be neglected.

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Note

High-performance liquid chromatographic procedures in monitoring the production and quality control of chlortetracycline

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Some tetracycline compounds, especially chlortetracycline, appear as more or less tailing peaks in different reversed-phase high-performance liquid chromatographic (HPLC) methods[1–3]. Reversed-phase column performance depends on the properties of the silica gel used and on the bonding technique. Because different reversed-phase packings are available, it is essential to select them carefully; a phase that is perfect for one separation may be inadequate for another. Performance also varies with the chromatographic conditions and the solutes under examination[4].

In this work, we tried to select the most suitable and simplest HPLC method for the precise and accurate determination of chlortetracycline and related tetracyclines that arise during biosynthesis in chlortetracycline production.

EXPERIMENTAL

Chromatographic system and methods

The isocratic mode was used for all experiments. The HPLC system consisted of an LKB 2150 pump, LKB 2151 variable-wavelength monitor, LKB 2220 computing integrator and a Rheodyne Model 7125 loop injector with a 20-μl fixed loop. Integration was based on peak-area measurement and determinations were carried out by the external standard method. Separations were made at ambient temperature. Degradation products of tetracycline and chlortetracycline were identified according to procedure of McCormick[5]. The columns used were LiChrosorb RP-8, 5-μm irregular particles (250 × 4.6 mm I.D.), TSK ODS 120 A, 5-μm particles (250 × 4.6 mm I.D.) (Merck, Darmstadt, F.R.G.), and Waters Nova-Pak C_{18} (75 × 3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.).

For different types of separations the following mobile phases and conditions were applied: 0.2 *M* citric acid + tetrabutylammonium hydroxide (0.5 g/l) (pH 2.5)–acetonitrile (92:8), UV detection at 360 nm; 0.05 *M* diethanolamine + 0.001 *M* disodium EDTA (pH 7.3)–isopropanol (88:12), UV detection at 360 nm; and methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:4), UV detection at 365 nm.

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Chemicals

Acetonitrile, methanol and tetrabutylammonium hydroxide (TBA) (20% aqueous solution) were obtained from Merck and oxalic acid dihydrate and sodium

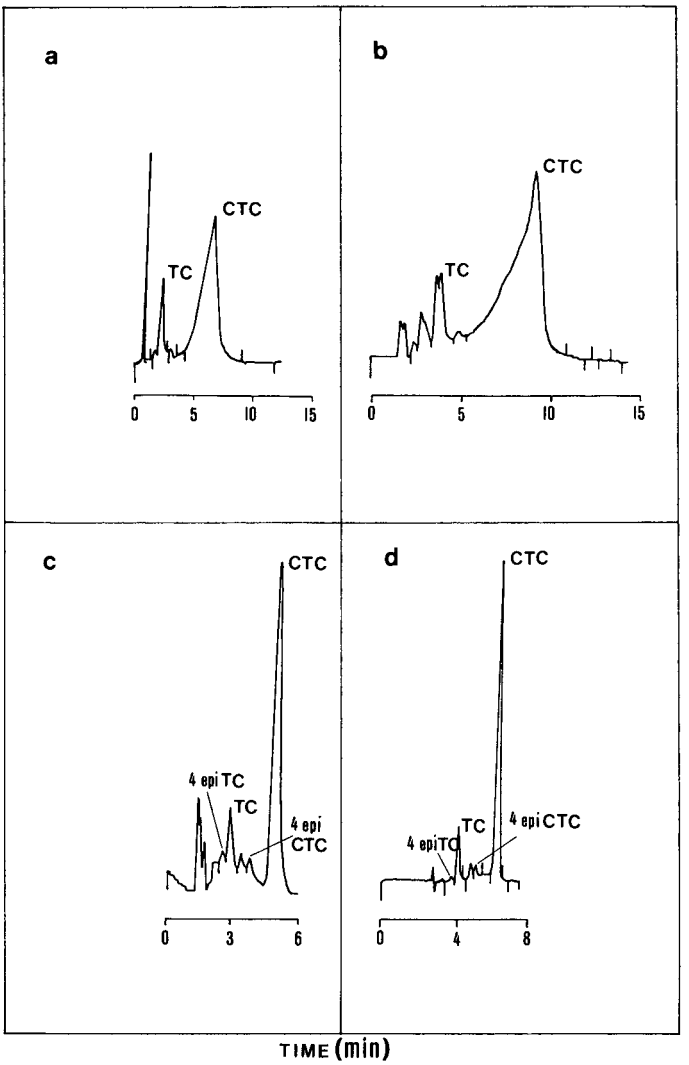

Fig. 1. Influence of different HPLC procedures on the separation of chlortetracycline referene standard. (a) Column, LiChrosorb RP-8, 5 μm (250 × 4.6 mm I.D.); mobile phase, 0.2 *M* citric acid + TBA (0.5 g/l) (pH 2.5)–acetonitrile (92:8); flow-rate, 1 ml/min; detection, UV at 360 nm. (b) Column, Waters Nova-Pak C_{18} (75 × 3.9 mm I.D.); mobile phase, 0.05 *M* diethanolamine + 0.001 *M* disodium EDTA (pH 7.3)–isopropanol (88:12); flow-rate, 0.5 ml/min; detection, UV at 360 nm. (c) Column, Waters Nova-Pak C_{18} (75 × 3.9 mm I.D.); mobile phase, methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:4); flow-rate, 0.5 ml/min; detection, UV at 365 nm. (d) Column, TSK ODS 120 A, 5 μm (250 × 4.6 mm I.D.); mobile phase, methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:4); flow-rate, 1 ml/min; detection, UV at 365 nm. Peaks: TC = tetracycline; 4-epi TC = 4-epitetracycline; CTC = chlortetracycline; 4-epi CTC = 4-epichlortetracycline.

ethylenediaminetetraacetate (EDTA) (both of analytical-reagent grade) from Kemika (Zagreb, Yugoslavia). Water for mobile phase preparation was deionized and distilled. Pure standards of chlortetracycline and tetracycline was obtained from the Institution for Drug Control and Testing (Zagreb, Yugoslavia). Different type of chlortetracycline samples were supplied by Krka Pharmaceuticals (Novo Mesto, Yugoslavia).

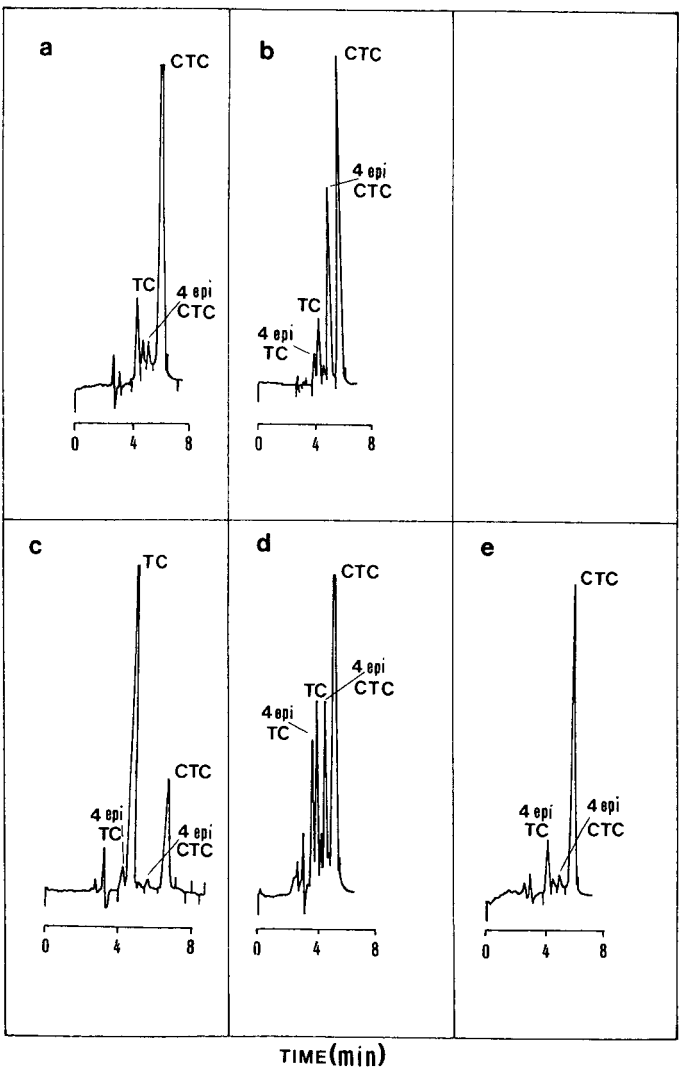

Fig. 2. HPLC separation of chlortetracycline by the optimum procedure. Column, LiChrosorb RP-8, 5 μm (250 × 4.6 mm I.D.); mobile phase, methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:4); flow-rate, 1 ml/min; detection, UV at 365 nm. Samples: (a) chlortetracycline reference standard; (b) chlortetracycline reference standard exposed to degradation procedure according to McCormick[5]; (c) chlortetracycline fermentation broth (low level of chlortetracycline); (d) chlortetracycline product (high level of 4-epichlortetracycline); (e) chlortetracycline product (proper quality, with level of 4-epichlortetracycline not higher than 5%). Abbreviations as in Fig. 1.

RESULTS AND DISCUSSION

In biotechnological processes for chlortetracycline production, a series of different tetracycline substances usually arise[6]. The fraction of tetracycline is produced simultaneously with chlortetracycline. The amounts of the degradation products of these two compounds such as 4-epitetracycline and 4-epichlortetracycline vary in relation to selected technological parameters. By using a suitable HPLC procedure, the appearance and monitoring of these compounds can be precisely studied during biotechnological processes.

For this purpose some different reversed-phase HPLC methods and columns were tested. It was found that the composition of the mobile phase played an important role in solving this problem[7]. In testing different types of reversed-phase column, it was found that columns of the highest quality and performances were not necessary if an appropriate mobile phase was chosen. Addition of TBA[3] and EDTA[1] to a particular mobile phase did not show the expected effects on tailing peaks (Fig. 1a and b). However, the addition of oxalic acid in an appropriate concentration acts as a very good complexation agent that prevents bonding of tetracycline substances to the reversed-phase material, thus preventing tailing effects[7] (Fig. 1c and d and Fig. 2).

The best results were obtained on a TSK ODS 120 A (5 μm) column (250 × 4.6 mm I.D.) and elution with methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:4) (Fig. 1d). The fully end-capped Waters Nova-Pak C_{18} column (75 × 3.9 mm I.D.) did not give a significant improvement in peak shape and resolution using the same mobile phase (Fig. 1c). The LiChrosorb RP-8 (5 μm) column with irregular particles (250 × 4.6 mm I.D.) gave satisfactory results, and this column with methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:4) as the mobile phase was selected for the simple and accurate monitoring of chlortetracycline production during the fermentation process and to establish quality control parameters for the final products (Fig. 2). With this method it proved possible to separate the tetracycline compounds and some of their degradation products in a relatively short time and with good symmetry of all peaks.

The appearance of the tetracycline fraction in the biosynthetic process is strongly reduced by the addition of a suitable concentration of Cu^{2+} together with Cl^- ions in the fermentation media. Simultaneously with reduction of tetracycline production, appropriate concentration of Cu^{2+} and Cl^-, induce high levels of chlortetracycline production[8] (Fig. 2b and c).

It is also possible to control the quality of chlortetracycline products with respect to the appearance of 4-epichlortetracycline as a degradation product and microbiologically inactive form of chlortetracycline. The epimerization of chlortetracycline to 4-epichlortetracycline[9] could be clearly demonstrated during the recovery procedure, whereas this process is not significant in the fermentation phase. Temperature seems to play an important role in the epimerization reaction, as this reaction is diminished when cooling is applied (Fig. 2d and e).

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Note

Investigation of the stability of 5-aminosalicylic acid in tablets and suppositories by high-performance liquid chromatography

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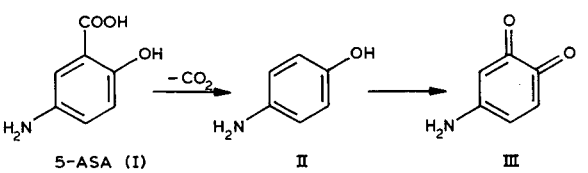
Mesalazine [Fisalamine; 5-aminosalicylic acid (5-ASA)] is a drug used in the treatment of Crohn's disease and ulcerative colitis[1,2] and is produced in two forms coated tablets and suppositories. 5-ASA is very unstable and it is necessary to ensure that a stable final preparation is obtained.

In this paper, an analytical method for the investigation of the stability of tablets and suppositories containing 5-ASA is described and the of influence of light and temperature on the stability of the pharmaceutical forms was studied. Generally, it was assumed that the same decomposition mechanism occurs for 5-ASA as for 4-aminosalicylic acid[3]:



5-ASA (I) II III

Compounds II and III are toxic and their presence in pharmaceutical forms must therefore be carefully controlled.

EXPERIMENTAL

Materials

Mesalazine (Nobel Chemicals, Sweden) was obtained as suppositories containing 250 mg of 5-ASA in 2 g and as tablets containing 250 mg of 5-ASA in 0.4 g.

Apparatus

A Shimadzu (Model UV-160) UV spectrophotometer was used. A modular liquid chromatograph was obtained from LKB, consisting of a Model 250 pump, a Model 2220 integrator, a Model 2151 variable-wavelength detector and a Rheodyne Model 7125 injector. The column (250 cm × 4 mm I.D.) was made of stainless steel and was packed with LiChrosorb RP-18 (10 μm).

Sample preparations for high-performance liquid chromatography (HPLC)

Tablets. A 160-mg amount of powdered tablets (which corresponds to *ca.* 100 mg of active substance) was extracted by shaking with 100 ml of mobile phase for 15 min, then the mixture was centrifuged and the supernatant liquid injected on to the column.

Suppositories. A 2-g amount of powdered suppositories was extracted by shaking with 100 ml of benzene for 15 min and filtered under vacuum. The filter-paper containing the precipitate was dried to constant weight at 40°C, 100 mg of the dry precipitate were dissolved in 100 ml of the mobile phase and portions of the solution were injected onto the column.

UV study of the influence of temperature on the stability of 5-ASA

It was observed visually that suppositories and tablets containing 5-ASA, on prolonged storage at elevated temperatures, changed colour, indicating a decomposition process. The decomposition process may be observed by UV spectrophotometry at 440 nm, because it results in an increase in absorbance at this wavelength (Table I).

Procedure. Dissolve 0.4 g of 5-ASA in a mixture of 10 ml each of ethanol and 0.5 *M* hydrochloric acid and measure the absorbance of the solution at 440 nm in a 1-cm cell.

HPLC investigation of the stability of 5-ASA in its pharmaceutical forms

Spectrophotometry is useful for the qualitative observation of the stability, but it is non-selective and hence unsatisfactory for the determination of the decomposition products. HPLC was therefore used for the investigation of the stability. Two separation systems were used, one to separate 5-ASA and salicylic acid (a by-product of the synthesis) and the second to separate 5-ASA and *p*-aminophenol.

System I. The mobile phase was acetonitrile–water (22:78, v/v) + 0.5% acetic acid at a flow-rate of 1 ml/min. Injections were made of 20 μl of a solution containing

TABLE I

STABILITY OF 5-AMINOSALICYLIC ACID IN SUPPOSITORIES AND TABLETS

Sample	*Time of storage (months)*	*Temperature (°C)*	*Absorbance at 440 nm*	*Appearance*
Tablets	0	Room	0.080	White
	6	Room	0.120	White
	12	Room	0.150	White
	6	37	0.357	Cream
	12	37	0.373	Cream
	6	45	0.367	Cream
	12	45	0.514	Cream
Suppositories	0	Room	0.123	White
	6	Room	0.200	White
	12	Room	0.235	White
	6	30	0.224	White
	12	30	0.283	White

TABLE II

DETERMINATION OF 5-AMINOSALICYLIC ACID IN SUPPOSITORIES AND TABLETS BY HPLC

Sample	*Time of storage (months)*	*Temperature (°C)*	*5-ASA (mg per tablet or suppository)*[a]	*Salicylic acid (%)*	*p-Aminophenol (%)*
Tablets	0	Room	251.2	0.1	–
	6	Room	251.0	0.1	–
	12	Room	250.8	0.1	–
	6	37	250.8	0.1	–
	12	37	250.7	0.1	–
	6	45	250.5	0.1	–
	12	45	249.7	0.1	–
Suppositories	0	Room	250.5	0.1	–
	6	Room	250.5	0.1	–
	12	Room	250.0	0.1	–
	6	30	249.8	0.1	–
	12	30	249.7	0.1	–

[a] Precision of the method: concentration of 5-ASA = 249.7–251.2 mg per tablet, mean = 250.85 and standard deviation = 0.589 (n = 9).

1 mg of active substance in 1 ml and 20 μl of a solution containing 0.004 mg of salicylic acid in 1 ml of the mobile phase. UV detection at 300 nm was used and the range was 0.04 a.u.f.s.

System II. The mobile phase was methanol–0.05 *M* NaH_2PO_4–0.025 *M* Na_2HPO_4–tetrabutylammonium phosphate (12:44:44:1, v/v) at a flow-rate of 0.6 ml/min. Injections were made of 20 μl of a solution containing 1 g of active substance and 20 μl of a solution containing 0.004 mg of *p*-aminophenol in 1 ml of the mobile phase. UV detection at 229 nm was used and the range was 0.04 a.u.f.s.

The external calibration method was used for the quantitative measurements, and the results are given in Table II.

RESULTS AND DISCUSSION

The analysis of suppositories and tablets by HPLC (system I) is shown in Fig. 1, where the peaks of the active substance (1) and salicylic acid (2) can be seen. The amount of salicylic acid is generally stable, because it is an intermediate in the synthesis of 5-ASA, its presence being due to inefficient purification of the final product. The analysis of the same products using system II (in which *p*-aminophenol can be determined) is shown in Fig. 2.

The results indicate that the tablets and suppositories examined contain no decomposition product, for which the retention time is 7.0 min. This is very surprising, because the presence of this compound as a contaminant was assumed according to the reaction shown. A compound with a retention time of 9.6 min was identified as sodium hydrosulphite ($Na_2S_2O_4$). Its amount is stable, because it is a raw material used in the synthesis. In tablets a peak having a retention time of 26 min was also found. It was identified as cellulose acetate phthalate, added as an excipient to tablets.

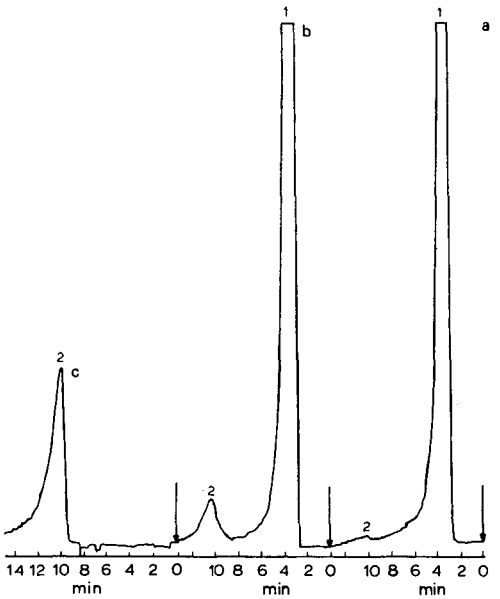


Fig. 1. Chromatograms of 5-ASA in (a) extract of tablets stored for 1 year at room temperature, (b) extract of tablets prepared from inefficient purification of 5-ASA and (c) a standard. Peaks: 1 = 5-ASA; 2 = salicylic acid.

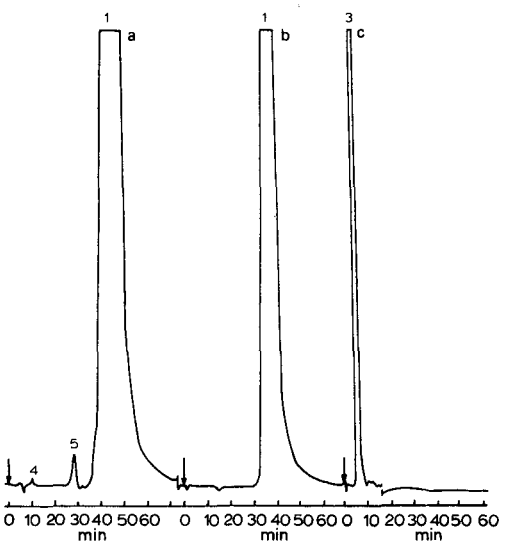


Fig. 2. Chromatograms of 5-ASA in (a) extract of tablets stored for 1 year at room temperature, (b) extract of suppositories stored for 1 year at room temperature and (c) a standard. Peaks: 1 = 5-ASA; 3 = *p*-aminophenol; 4 = sodium hydrosulphite; 5 = cellulose·acetate phthalate.

The results indicate that the tablets and suppositories containing 5-ASA are sufficiently stable. The decrease in the content of the active substance in these forms stored at room temperature for 1 year does no exceed 1% and the content of salicylic acid is 0.1%. The HPLC method is sufficiently selective and sensitive for controlling the stability of pharmaceutical forms of 5-ASA.

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Chromatographic separation of surfactants

XII. Quantification of results in trial high-performance liquid chromatography of ethoxylated surfactants

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ABSTRACT

The high-performance liquid chromatographic (HPLC) analysis of the oligomer distribution of ethoxylated alkylphenols was checked by interlaboratory testing on three samples with low degrees of ethoxylation in ten laboratories. Results for the oligomer composition showed good accuracy, repeatability and reproducibility and indicated that differences in instrumentation, operating conditions and operators' experience did not influence the results. The peaks areas were corrected by different factors depending on whether refractive index or UV detection was used. The mean molecular masses and degrees of ethoxylation calculated from the HPLC data were in very good agreement with indicated or theoretical values, demonstrating the reliability of the HPLC results. Results of thin-layer chromatographic analysis of the same samples with flame ionization detection were very similar to those obtained by HPLC.

INTRODUCTION

The quantitative evalutation of chromatograms obtained by high-performance liquid chromatography (HPLC) of ethoxylated surfactants is a problem. In recent publications mainly separation problems were discussed, only limited attention being devoted to the quantitative evaluation of results[1]. The important requirements for the quantitative evaluation of the composition of oligomer adducts in ethoxylated surfactants are the following: to ensure complete recording of all the oligomers present in the sample; to achieve a sufficient separation of all recorded oligomer peaks; to be certain of their identification; and to have confidence at least in the relative responses of the detection method applied.

To meet these requirements, various ethoxylated surfactants have been analysed by HPLC in our laboratory in recent years applying diol bonded phases and *n*-hexane–2-propanol–water mobile phases[2,3]. The proportions of the components in these mobile phases were adjusted so as to give as complete a separation of oligomers

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as possible in samples of different degrees of ethoxylation with isocratic elution using refractive index (RI) detection. For identification of oligomers in ethoxylated fatty alcohols some pure synthetic standards of lower oligomers were available. Oligomers in other ethoxylates were identified without standards, relying on the sequence continuity of oligomers starting from samples with the lowest degree of ethoxylation.

For the correction of oligomer peaks area with refractive index (RI) detection, relative correction factors were calculated from the differences in n_D values between separate oligomers and the mobile phase applied[3,4]. Appropriate n_D data for ethoxylated surfactants and their oligomers were taken from the literature[5,6] and in some instances were estimated from n_D measurements on samples with different degrees of ethoxylation assuming a simple, smooth dependence of n_D on degree of ethoxylation in order to make inter- and extrapolations of n_D data for individual oligomers.

For UV detection it was found the molar absorptivities of ethoxylated alkylphenols were independent on the polyoxirane chain length[7–10], so that the chromatograms obtained showed the molar distribution of oligomers. Other detection methods for the quantification of ethoxylated surfactants do not appear to have been published.

In spite of numerous applications of HPLC to the separation of ethoxylated surfactants, data on the repeatability and reproducibility are lacking. Only in one paper[11] were coefficients of variation published for nineteen oligomers of ethoxylated alkylphenols. To consider the reliability, accuracy, repeatability and reproducibility of HPLC separations of ethoxylated surfactant oligomers, interlaboratory testing was performed with samples of alkylphenols of low degree of ethoxylation. The choice of samples was limited by the instrumentation and experience available in the participating laboratories and by the effort of analyzing possibly simple samples with a reduced number of oligomers under analogous operating conditions to show the ability of HPLC to differentiate samples with only small variations in their degree of ethoxylation. For the establishment of optimum conditions for interlaboratory testing, preliminary tests in authors laboratory were performed under a wide range of operating conditions with different columns and mobile phase compositions with RI and UV detection. On the basis of this experience, directions and recommendations were despatched to the participating laboratories with three samples. The results of this interlaboratory testing are presented in this paper.

EXPERIMENTAL

HPLC instrumentation

The instrumentation used in the ten participating laboratories is summarized in the Table I. The results were evaluated from the oligomer peaks areas, applying different corrections for RI and UV detection as indicated in the Introduction; correction factors for the evaluation of the mass distribution are given in the Table II. For RI detection they were evaluated from differences in n_D between oligomers[5,6] and the mobile phase; correction factors for UV detection were calculated from comparison of corrected results for RI detection and the peak-area composition with UV detection at 258 nm. Theoretical correction factors for UV detection are also indicated, calculated from the corresponding molecular weights presuming that UV detection gave the molar composition.

TABLE I

HPLC INSTRUMENTATION IN INTERLABORATORY TESTING

Laboratory No.	*Instrument*	*Injector*	*Detector*		*Integrator*
			RI	*UV*	
1	HPP 4001	Rheodyne 7125	RIDK 101	UVD LCD, 254 nm	SP Minigrator
2	SP 8750	SP 8700	–	SP 8440, 258 nm	SP 4270
3	HP 1050	Autosampler	–	UVD, 230 nm	HP 1090 Workstation
4	SP 8100	Valco	–	LCD 2563, 254 nm	SP 4100
5	HPP 4001	LCI 02 septum	RIDK 102	–	CI 100
6	HPP 4001	Stop-flow	RIDK 101	–	–
7	HPP 4001	LCI 02 septum	RIDK 101	–	–
8	PU 4100	Rheodyne 7125	–	PU 4110, 280 nm	PU 4810
9	Knauer FR-30	Rheodyne 7120	Knauer 61.00	Knauer, 258 nm	Chromatopac CR-3-A
10	HPP 5001	Stop-flow	RIDK 101	–	CI 100

Thin-layer chromatography (TLC) with flame ionization detection (FID)

Parallel TLC–FID analyses[12] were performed on an Iatroscan TH-10 Mark III instrument using Chromarod S II for separations with stepwise development using two mobile phases: (a) benzene–ethyl acetate (6:4, v/v) to a distance of 10 cm from the start and (b) ethyl acetate–acetic acid–water (8:1:1, v/v/v) up to a distance of 8 cm. Samples were applied as 2% solutions in chloroform and a 1-μl volume of the solution was applied to one Chromarod at the start. FID was operated with hydrogen at

TABLE II

CORRECTION FACTORS FOR RI AND UV DETECTION AT 258 nm RELATIVE TO OLIGOMER 5

Oligomer No.	*RI detection*	*UV detection*			
		For dodecylphenols		*For nonylphenols*	
		Theory	*Found*	*Theory*	*Found*
1	0.921	0.635	0.721	0.600	0.578
2	0.949	0.726	0.789	0.700	0.712
3	0.969	0.817	0.860	0.800	0.811
4	0.986	0.909	0.933	0.900	0.907
5	1.000	1.000	1.000	1.000	1.000
6	1.013	1.091	1.066	1.100	1.090
7	1.026	1.182	1.136	1.200	1.178
8	1.039	1.274	1.201	1.300	1.266
9	1.051	1.249	1.266	1.400	1.351
10	1.063	1.340	1.331	1.500	1.447
11	1.073	1.548	1.393	1.600	1.558
12	1.083	1.639	1.462	1.700	1.689
13	1.091	1.730	1.526	1.800	1.842
14	1.099	–	–	1.900	2.026
15	1.107	–	–	2.000	2.249
16	1.114	–	–	2.100	2.533

160 ml/min and air at 2200 ml/min. The scanning time was 35 s for each Chromarod. The results were evaluated from the peak areas of the oligomers, applying correction factors considering the relative contributions of different carbon atoms in the molecular structure to the FID response[13], analogous to those applied for the TLC–FID of dodecyl ethoxylates[14].

Conditions for interlaboratory testing

In the directions to the various laboratories, the application of diol bonded phases and RI or UV detection under isocratic conditions or gradient elution with UV detection was recommended, applying *n*-hexane–2-propanol–water mobile phases. The aim was to achieve an acceptable separation of oligomers within the complete distribution by the choice of a suitable mobile phase composition. Provision of at least three complete results for the mass composition of oligomers from subsequent analyses of each of the three samples was requested. The HPLC operating conditions applied in individual laboratories are summarized in the Table III.

Samples

The properties of the samples used are given in Table IV. All other chemicals and solvents used were of analytical-reagent grade.

TABLE III

OPERATING CONDITIONS IN INTERLABORATORY TESTING

Laboratory No.	*Sorbent in 250 × 4 mm I.D. column and grain size*	*n-Hexane–2-propanol–water mobile phase (v/v/v)*	*Flow-rate (ml/min)*	*Sample volume (μl)*	*Sample concentration (%)*
1	Silasorb Diol, 4.7 μm	82:18:0.03	0.8	10	4–5
2	LiChrosorb Diol, 5 μm	75:25:1 or gradient from 90:10: saturated to 70:30:1 in 30 min	0.6	10	2–7
3	Silasorb SPH Amin 10 μm	gradient from 100:0:0 to 0:100:0 in 65 min	1.0	20 or 40	2–5
4	LiChrosorb Diol, 5 μm	82:18:0.03	1.0	10	1
5	LiChrosorb Diol, 5 μm	75:25:1	0.4 or 0.8	3–8	> 10
6	LiChrosorb Diol, 5 μm	82:18:0.03	0.5	1–5	20
7	LiChrosorb Diol, 5 μm	70:30:2 for higher and 82:18:0.03 for lower oligomers	0.5	20–25	5–6
8	LiChrosorb Diol, 5 μm	gradient from 90:10:saturated to 75:25:1 in 10 min, then 15 min isocratic	1.0	5	*ca.* 8
9	LiChrosorb Diol, 5 μm	90:10:saturated for lower and 75:25:1 for higher oligomers	0.8	20	2–5
10	LiChrosorb Diol, 5 μm	82:18:0.03	0.8	20	2–5

TABLE IV

SAMPLES ANALYSED IN INTERLABORATORY TESTING

Property	*Dodecylphenol + 6 EO*	*Slovafol 905*	*Emulgator U 6*
Origin	Laboratory sample	Chemical plant, Nováky, Czechoslovakia	Unger, Norway
Designation	DDF	S 905	U 6
Hydrophobe	Dodecylphenol	Nonylphenol	Nonylphenol
Degree of ethoxylation mol EO/mol	6.0	5.0	6.0
Sample composition:			
Free PEG (%)	3.3	2.4	1.2
Free alkylphenol (%)	0.3	0.2	0.1
Oligomer adducts (%)	96.4	97.4	98.7

RESULTS

The data obtained by all the participating laboratories are very numerous and cannot be presented in full. Using isocratic conditions, in six laboratories RI detection and in four laboratories UV detection were applied; gradient elution with UV detection was used in three laboratories. These three groups of mean HPLC results obtained under different conditions are given for the three samples in Tables V–VII together with the TLC–FID results and data for mean molecular mass calculated from these composition data. Some chromatograms produced by the participating laboratories are given in Figs. 1–4.

TABLE V

MEAN COMPOSITION OF DDF AND CORRESPONDING MEAN MOLECULAR MASS DATA

Oligomer No.	*RI detection in 6 labs.*	*UV detection in 4 labs.*	*Gradient elution in 3 labs.*	*TLC–FID in 1 lab.*
1	0.7	0.7	3.6	0.3
2	3.4	3.8		2.2
3	8.9	9.2	8.6	8.9
4	14.5	14.9	14.3	15.2
5	17.1	17.3	18.8	20.0
6	16.1	16.1	15.4	17.7
7	13.6	13.3	13.4	14.8
8	10.4	10.3	10.7	8.7
9	7.0	6.7	6.9	6.1
10	4.4	3.9	4.1	3.1
11	2.3	1.9	2.3	1.8
12	1.2	1.1	1.2	0.9
13	0.4	0.8	0.7	0.3
Mean molecular mass	509.4	506.3	511.0	507.0

TABLE VI

MEAN COMPOSITION OF S 905 AND CORRESPONDING MEAN MOLECULAR MASS DATA

Oligomer No.	*RI detection in 6 labs.*	*UV detection in 4 labs.*	*Gradient elution in 3 labs.*	*TLC–FID in 1 lab.*
1	1.0	1.1	1.0	0.4
2	6.2	6.8	6.2	5.7
3	15.4	15.6	14.6	19.5
4	20.8	20.4	19.1	24.4
5	18.9	18.9	18.6	20.9
6	14.4	14.3	15.4	13.5
7	9.8	9.8	10.4	8.4
8	6.2	6.1	6.7	3.7
9	3.6	3.3	3.7	2.0
10	1.7	1.7	2.2	1.0
11	1.1	1.0	1.2	0.5
12	0.6	0.8	0.6	–
13	0.3	0.3	0.3	–
Mean molecular mass	429.1	427.3	432.8	416.6

The data from repeated HPLC analyses in individual laboratories were checked for standard deviations and corresponding coefficients of variation by appropriate calculations[15]. The calculation of the repeatability of the results (in individual laboratories) and reproducibility (between laboratories) was carried out according to the recommended standard[16]. No outlying values were found. The statistical tests[15] for

TABLE VII

MEAN COMPOSITION OF U 6 AND CORRESPONDING MEAN MOLECULAR MASS DATA

Oligomer No.	*RI detection in 6 labs.*	*UV detection in 4 labs.*	*Gradient elution in 3 labs.*	*TLC–FID in 1 lab.*
1	0.6	0.7	0.6	0.2
2	3.2	3.5	3.2	1.7
3	8.1	8.6	7.6	7.1
4	13.1	13.3	12.3	13.8
5	14.5	14.7	13.9	15.7
6	14.4	14.5	14.3	16.0
7	13.3	13.3	13.4	14.9
8	11.3	11.1	11.9	11.2
9	8.3	7.9	9.2	7.5
10	5.4	5.2	6.0	5.3
11	3.6	3.1	3.6	3.2
12	2.1	2.0	2.1	2.1
13	1.0	1.0	1.4	0.9
14	0.6	0.6	0.3	0.4
15	0.3	0.3	0.2	–
16	0.2	0.2	–	–
Mean molecular mass	480.1	476.0	483.9	483.5

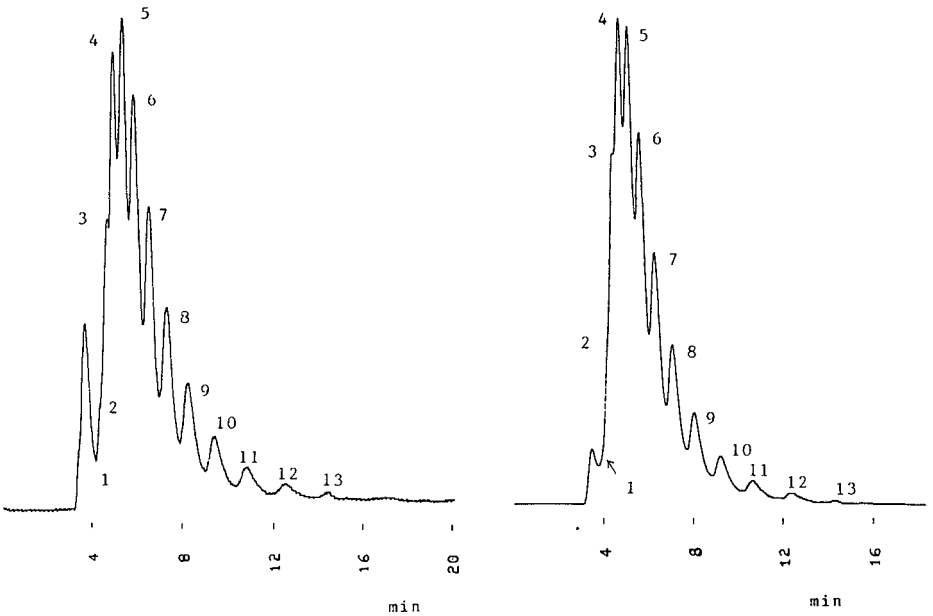


Fig. 1. Separation of DDF with (left) RI and (right) UV detection in laboratory No. 9 using mobile phase with ratio of components 75:25:1; oligomers 1–13.

differences between RI and UV detection with isocratic and gradient elution showed criteria without any significance, *i.e.*, the differences in the results are not caused by differences in the chromatographic variants used.

A survey of the resulting statistical data is presented in Table VIII, grouped according to the oligomers content. The dependence of the relative repeatability and relative reproducibility on the oligomer content is shown graphically in Figs. 5 and 6.

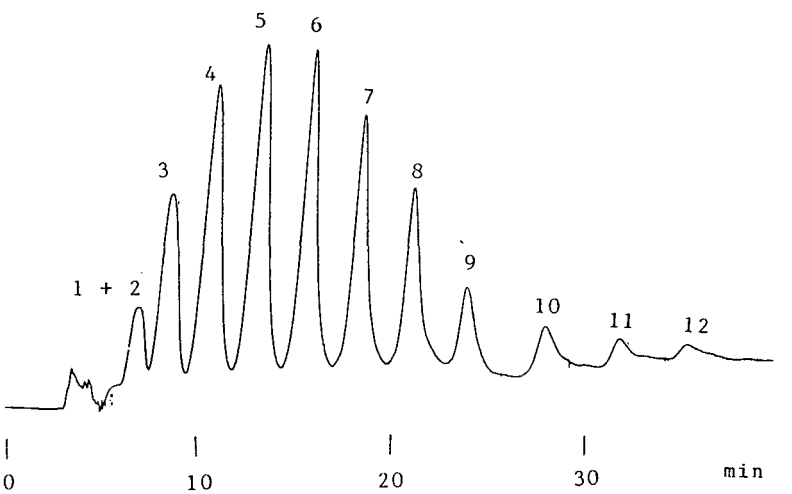


Fig. 2. Separation of DDF in laboratory No. 2 using gradient elution with UV detection; oligomers 1–12.

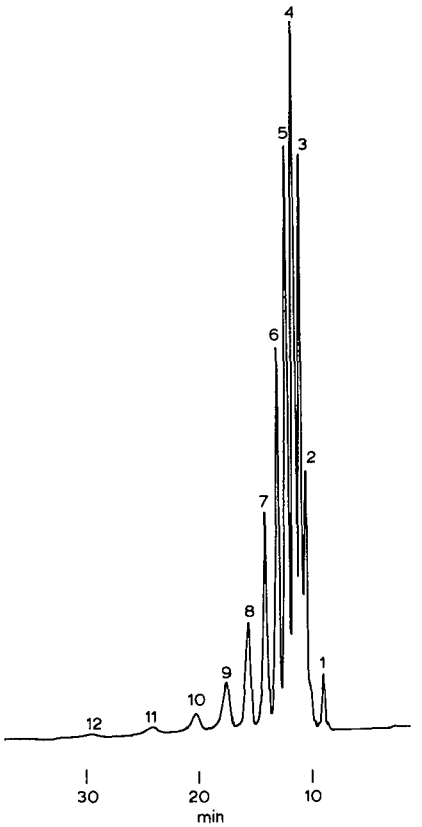


Fig. 3. Separation of S 905 in laboratory No. 5 using mobile phase with ratio of components 75:25:1; oligomers 1–12.

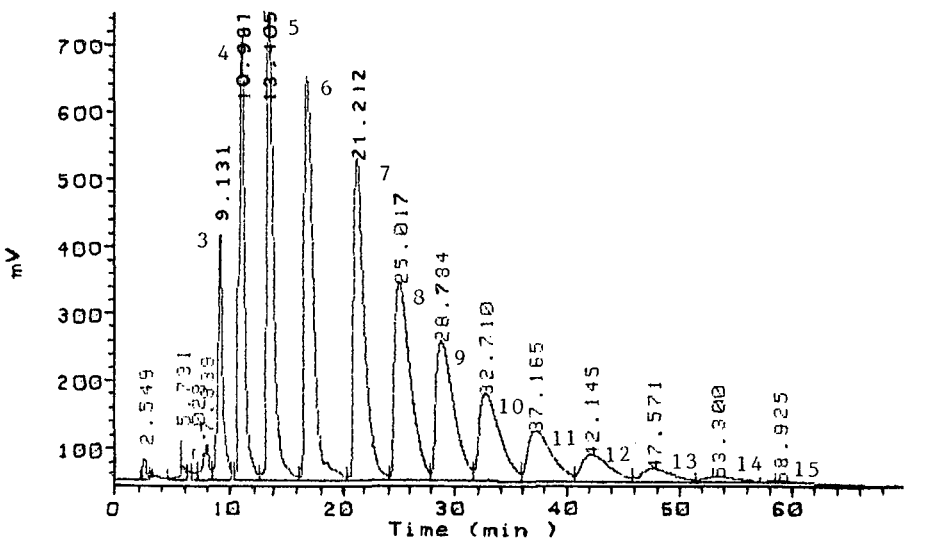


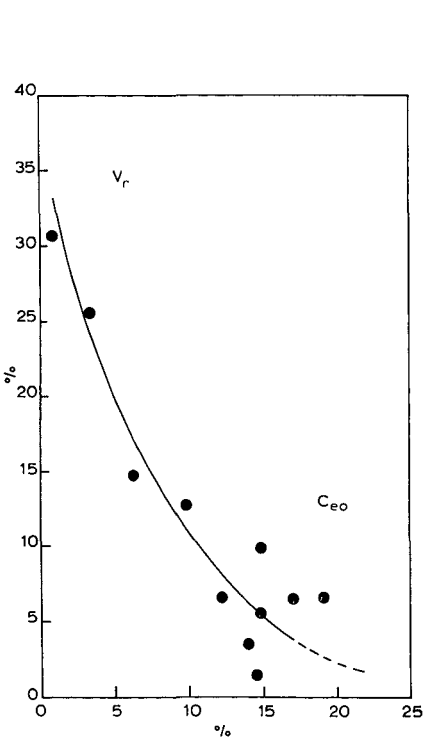
Fig. 4. Separation of U 6 in laboratory No. 3 with linear gradient elution from 100% *n*-hexane to 100% 2-propanol in 65 min with UV detection at 230 nm; up to oligomer 15.

TABLE VIII

STATISTICAL DATA FROM INTERLABORATORY TESTING

Parameter	*Main component oligomers*	*Other substantial oligomers*	*Trace component oligomers*
Contents (%)	13–20	3–12	⩽3
Standard deviation[a]	0.2–0.8	0.4–1.3	>0.3
Coefficient of variation (%)	1–6	6–12	>20
Repeatability	0.2–1.2	0.9–1.3	>0.3
As % of result	1.3–6.5	8–15	>30
Reproducibility	1.1–1.7	1.0–1.7	>0.3
As % of result	9–12	13–20	>40

[a] In separate oligomers content determinations.



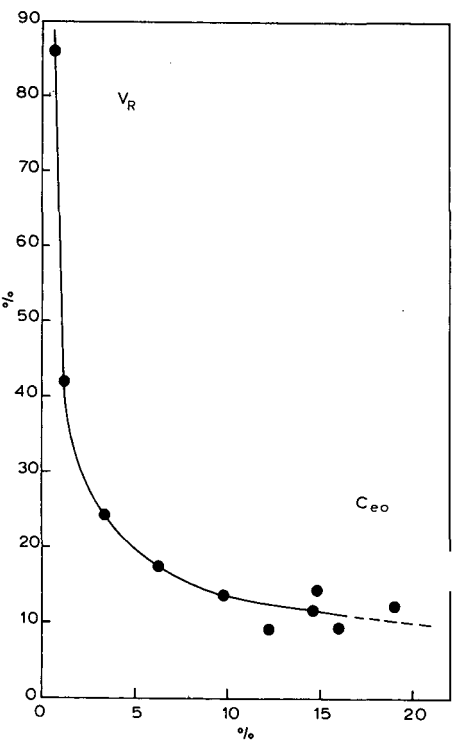


Fig. 5. Dependence of relative repeatability (v_r) in HPLC on relative contents of EO oligomers (c_{eo}).

Fig. 6. Dependence of relative reproducibility (v_R) in HPLC on relative contents of EO oligomers (c_{eo}).

DISCUSSION

From the mean results of oligomer mass composition in Tables V–VII it is evident that the three HPLC variants gave nearly identical results with respect to distribution and contents of oligomers. This conformity of results is very valuable for confirming the efficient performance of the analysts in the participating laboratories and an effective arrangement for interlaboratory testing. In spite of the differences in instrumentation, operating conditions and HPLC experience among the laboratories, concordant data were obtained, and the results were not influenced by these differences evidently. In addition, almost identical results were obtained by an independent method, TLC–FID, where the separation of the oligomers was comparable to the HPLC separation with gradient elution, *i.e.*, better than HPLC with isocratic elution[17].

The statistical data (Table VIII) and their dependence on oligomer content (Figs. 5 and 6) showed repeatability values usual in chromatographic practice, *i.e.*, 5% relative or better for the main components, higher for other components and over 20% for trace components; data for relative reproducibility indicate a coincidence between two laboratories of within *ca.* 10% for the results for the main component oligomers, better than 20% for other constituents and worse than 40% for trace components. In this respect the results of interlaboratory testing are satisfactory.

Using the composition data for the calculation of mean molecular mass (MMM) of the corresponding surfactants (Tables V–VII), an even better coincidence is evident (within 1–3% from MMM data), showing the excellent accuracy of the HPLC analyses. Nearly, the same level of coincidence was found between theoretical MMM data calculated from the indicated degree of ethoxylation and MMM data calculated from the mean HPLC data (Table IX) for separate samples. All the HPLC data are lower, however, than those calculated from the degree of ethoxylation; this is evidently caused by the presence of free polyethylene glycol (PEG) in the samples (Table III). When the indicated degree of ethoxylation of the samples is compared with values calculated from the HPLC results considering the free PEG contents, nearly identical values were found (Table X). Thus the reliability of HPLC results is confirmed also by this way.

The capability of HPLC to differentiate between samples with different degrees of ethoxylation is clear; even differences substantially lower than 1 mol EO/mol can be determined owing to the reliability of HPLC.

The results indicate that all the requirements outlined in the introduction for quantitative evaluation in the HPLC analysis of ethoxylates were satisfied and the goals of interlaboratory testing were also met. Complete distributions of oligomers

TABLE IX

MEAN MOLECULAR MASS (MMM) DATA OF ADDUCTS IN ANALYSED SAMPLES

MMM	*DDF*	*S 905*	*U 6*
Theoretical MMM from degree of ethoxylation	526.8	426.6	484.7
Calculated MMM from mean HPLC data	508.3	428.3	478.5

TABLE X

DEGREE OF ETHOXYLATION OF ANALYSED SAMPLES (mol EO/mol)

Degree of ethoxylation	*DDF*	*S 905*	*U 6*
Indicated	6.0	5.0	6.0
Calculated from mean HPLC data, *i.e.*, for adducts	5.64	4.77	5.89
Corrected for free PEG content	5.98	5.00	6.00

were attained and their HPLC peaks were sufficiently separated and identified without difficulty. The applied correction factors for RI and UV detection appeared to be appropriate, giving reliable results; the differentiation between samples with slight variations in their degree of ethoxylation was easy by HPLC.

CONCLUSIONS

The oligomer distribution in ethoxylated surfactants of the alkylphenol type with lower degrees of ethoxylation can be determined by HPLC separation on diol bonded phases using RI or UV detection under isocratic conditions or with UV detection under gradient elution with *n*-hexane–2-propanol–water mobile phases. The results showed high reliability and acceptable accuracy, repeatability and reproducibility. The mean molecular mass data and degree of ethoxylation of the samples analysed can be calculated from the HPLC results, showing even better coincidence with the theoretical values. The coincident results of parallel TLC–FID analyses support the reliability of the HPLC results.

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High-performance gel permeation chromatography of water-soluble β-1,3-glucans

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ABSTRACT

High-performance gel permeation chromatography of water-soluble β-1,3-glucans was carried out on Separon HEMA-S as the sorbent in 0.1 *M* sodium chloride solution as the mobile phase at 25°C. This system was calibrated with two sets of standards. The molecular-weight distributions of the fractions of the sodium salt of carboxymethyl-β-1,3-glucan (CMG-Na), schizophyllan and lentinan and of the same samples treated with dimethyl sulphoxide were determined. The results obtained were almost identical. The only difference was found in the case of schizophyllan owing to melting of its higher order structure.

INTRODUCTION

Glucans are natural biopolymers, polysaccharides with biological effects on the living body[1–3]. The water-soluble β-1,3-glucans, schizophyllan[4] and lentinan, are produced commercially in the injection form.

Studies of the behaviour of water-soluble β-1,3-glucans and/or their derivatives in solution are very sparse. Although the melting and degradation of the higher order structure of schizophyllan at various treating conditions have been mentioned[5–9], systematic high-performance gel permeation chromatographic (HPGPC) studies in this field have not yet been reported. However, checks of polymer stability during long-term storage either in powdered form or in solution are important. Moreover, sterilization of the polymer injection form by heating or irradiation may influence the

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molecular-weight distribution of β-1,3-glucans. HPGPC could be very effective in revealing such changes.

In previous work, β-1,3-glucan from cell walls of the yeast *Saccharomyces cerevisiae* was used for the preparation of a water-soluble derivative, the sodium salt of carboxymethyl-(1→6)-β-D-gluco-(1→3)-β-D-glucan (CMG-Na)[10]. CMG-Na was fractionated[11] and characterized by combined methods of gel permeation chromatography, light scattering and capillary viscometry[12].

In this study we studied the properties of CMG-Na samples by HPGPC. We determined the molecular-weight distribution and compared the behaviour of natural polymeric samples with that of samples treated with dimethyl sulphoxide (DMSO). Commercial samples of lentinan and schizophyllan were treated under the same conditions.

EXPERIMENTAL

Materials

TSK standards (Toyo Soda, Tokyo, Japan) of poly(ethylene oxide) (PEO) having molecular weights of $2.1 \cdot 10^4$ (SE-2), $4.5 \cdot 10^4$ (SE-5), $8.5 \cdot 10^4$ (SE-8) were used. Hydroxyethyl-starches were a gift from Dr. Kirsti Granath (Pharmacia, Uppsala, Sweden (weight-average molecular weight, $\bar{M}_w = 5.38 \cdot 10^4$, $1.28 \cdot 10^5$, $1.95 \cdot 10^5$, $3.98 \cdot 10^5$). Other chemicals used were of the analytical-reagent grade, except DMSO (spectroscopic grade). Lentinan (1 mg of lyophilizate) and schizophyllan (20 mg in 2 ml; clinical grade) were purchased from Taito (Kobe, Japan). Fractions of the sodium salt of carboxymethyl-(1→6)-β-D-gluco-(1→3)-β-D-glucan (II, $4A_2$, 8 and III) were prepared by stepwise precipitation using acetone[12].

High-performance gel permeation chromatography

The HPGPC experiments were performed with a high-pressure pump (HPP 5001, Laboratorní přístroje, Prague, Czechoslovakia), an eight-port switching valve equipped with two 100-μl loops (Model PK 1, Vývojové dílny, Czechoslovak Academy of Sciences, Prague, Czechoslovakia), two stainless-steel columns (250 × 8 mm I.D.) packed with Separon HEMA-S 1000 and Separon HEMA-S 300 (mean particle size 10 μm) connected in series (Tessek, Prague, Czechoslovakia) and a differential refractometric detector (RIDK 102, Laboratorní přístroje). Experiments were carried out at 25°C. The mobile phase was 0.1 *M* sodium chloride solution. The flow-rate of the eluent, "degassed" by purging with helium, was constant at 0.7 ml/min. The sample volume injected was 100 μl and the sample concentration was 0.5%.

Preparation of sample solutions for HPGPC

The CMG-Na fractions, schizophyllan and lentinan were dissolved in the mobile phase.

All samples (2.5 mg each) were treated with DMSO (0.5 ml). The samples were dissolved in DMSO overnight, then evaporated under nitrogen at 50°C. The last evaporation step took *ca.* 4 h. Each solid polymer was dissolved in the mobile phase (0.5 ml) prior to HPGPC analysis.

RESULTS AND DISCUSSION

The equipment for HPGPC was calibrated with two sets of standards, *viz.*, PEOs and hydroxyethyl-starches. Because the high-molecular-weight PEO standards showed strong hydrophobic interaction effects with the sorbent, only low-molecular-weight materials were used. The $\bar{M}_w$ value reported by the producer was taken as M (peak). The dependences of the elution volume (V_e) on the logarithm of molecular weight (M) for both reference polymers are linear (Fig. 1) in the molecular-weight range measured:

for hydroxyethyl starch:

$$\ln M = 19.3663 - 0.03417V_e \qquad (r = 0.9989)$$

for PEO:

$$\ln M = 15.4639 - 0.01874V_e \qquad (r = 0.9987)$$

where V_e is in counts.

Correction for the broadening effect was made mathematically[13] by solving Tung's integral equation[14]. The instrumental spreading function was approximated by the Gaussian curve with a resolution factor h. The values of h were determined by the procedure proposed by Balke and Hamielec[15], using their relationship:

$$\bar{M}_w/\bar{M}_n = \bar{M}_w^*/\bar{M}_n^* \exp(-D_2^2/2h)$$

where $\bar{M}_w^*$ *and* $\bar{M}_n^*$ are the weight- and number-average molecular weights calculated from the uncorrected molecular-weight distribution curve and D_2 is the slope of the calibration graphs described by the equation $\ln M = D_1 - D_2V_e$.

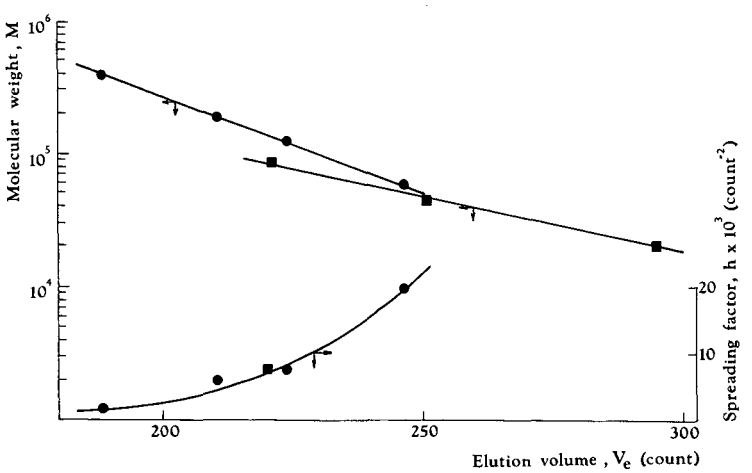

Fig. 1. Calibration of the HPGPC equipment. ● = Hydroxyethyl-starch; ■ = PEO.

TABLE I

CALIBRATION DATA OF THE HPGPC INSTRUMENT RESOLUTION

Specification of reference samples		*Calculated values*			
$\bar{M}_w$	$\bar{M}_w/\bar{M}_n$	$\bar{M}_w^*/\bar{M}_n^*$	$h \times 10^3$ (counts^{-2})	$\bar{M}_w$	$\bar{M}_w/\bar{M}_n$
Hydroxyethyl-starch:					
$3.98 \cdot 10^5$	1.276	1.832	1.61	$3.94 \cdot 10^5$	1.391
$1.95 \cdot 10^5$	1.204	1.334	5.69	$2.08 \cdot 10^5$	1.209
$1.28 \cdot 10^5$	1.196	1.294	7.41	$1.47 \cdot 10^5$	1.239
$5.38 \cdot 10^4$	1.338	1.378	19.81	$7.60 \cdot 10^4$	1.351
Poly(ethylene oxide):					
$8.50 \cdot 10^4$	1.06	1.085	7.53	$8.36 \cdot 10^4$	1.069
$4.50 \cdot 10^4$	1.07	1.203	1.50	$4.83 \cdot 10^4$	1.201
$2.10 \cdot 10^4$	1.12	1.117	Negative	$2.18 \cdot 10^4$	1.114

The relationship $h = f(V_e)$ was fitted by a polynomial of the second order:

$$h = 0.1755 - 1.8547 \cdot 10^{-3} V_e + 4.9537 \cdot 10^{-6} V_e^2 \qquad (r = 0.9929)$$

for samples of hydroxyethyl-starch and PEO, where h is in counts^{-2}. The iterative program of Chang and Huang[16] was used for calculation of both uncorrected and corrected averages of the molecular weight of all samples.

Table I contains the calibration data for the HPGPC instrument for "imperfect" resolution. On comparing the values of $\bar{M}_w^*/\bar{M}_n^*$ for each reference polymer given in Table I with the value of $\bar{M}_w/\bar{M}_n$ calculated from the corrected molecular-weight distribution curve, it is evident that the intensity of the sample broadening during HPGPC is not so high and both values are almost identical, as is typical in high-performance chromatography. The method for the calculation of the corrected

TABLE II

HPGPC DISTRIBUTION ANALYSIS OF POLYMERIC SAMPLES

Distribution analysis was done by using the calibration dependence $M = f(V_e)$ for hydroxyethyl-starch (see also Fig. 1).

Sample	*Natural polymeric sample*		*Sample treated with DMSO*	
	$\bar{M}_w$	$\bar{M}_w/\bar{M}_n$	$\bar{M}_w$	$\bar{M}_w/\bar{M}_n$
Schizophyllan	$6.24 \cdot 10^5$	1.307	See Fig. 2	
Lentinan	$2.66 \cdot 10^5$	3.705	$2.05 \cdot 10^5$	3.226
CMG-Na fraction:				
II	$7.63 \cdot 10^5$	1.712	$6.41 \cdot 10^5$	1.395
$4A_2$	$7.04 \cdot 10^5$	1.163	$6.59 \cdot 10^5$	1.231
8	$5.28 \cdot 10^5$	1.631	$5.10 \cdot 10^5$	1.640
III	$1.32 \cdot 10^5$	2.441	$9.71 \cdot 10^4$	1.727

molecular-weight distribution gives real values of $\bar{M}_w$ and $\bar{M}_n$ for the calibration standards (Table I).

With regard to the ionic character of the CMG-Na samples, 0.1 *M* sodium chloride solution was used as the mobile phase[17]. In contrast to the work of Kato *et al.*[18], the universal calibration graph is not valid for the standards used here, probably owing to their hydrophobic interaction with the Separon HEMA-S sorbent. Therefore, the β-1,3-glucan chromatograms were further treated by using the set of calibration data $M = f(V_e)$ valid for hydroxyethyl-starch. This is why the calculated molecular weight averages of these samples given in Table II should be taken as relative.

The β-1,3-glucan schizophyllan is considered to be exceptional in that in water its macromolecules adopt a higher order structure of a triple helix. From the crystallographic data, the same triple-helical conformation may be suggested for lentinan in the solid state. A number of papers[5–9] had shown that under denaturing conditions (alkaline pH, heating, admixing of DMSO with an aqueous solution of schizophyllan), the higher order structure of schizophyllan is probably unfolded.

We have studied the CMG-Na fractions, lentinan and schizophyllan after treatment with DMSO at higher temperature (50°C). Schizophyllan dissolves very well in DMSO and other β-1,3-glucan fractions of CMG-Na swell. Such treatment caused a total melting effect of schizophyllan, evident from the disappearance of the sample signal at low V_e (Fig. 2), whereas the other β-1,3-glucans, i.e., lentinan and the CMG-Na fractions, were not been affected by the DMSO treatment (Table II). Consequently, the undirected molecular-weight distribution analysis neither excludes nor supports the triple-helical structure of the CMG-Na fractions and of lentinan in aqueous solution.

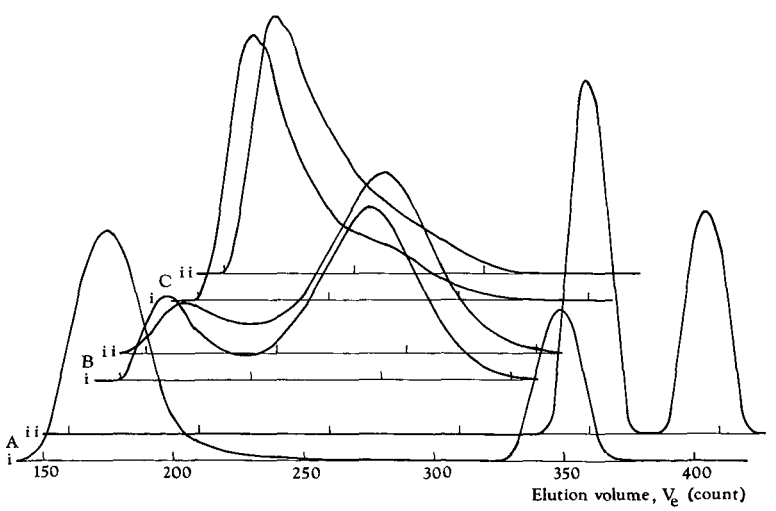

Fig. 2. Normalized chromatograms of (A) schizophyllan, (B) lentinan and (C) carboxymethylglucan II fraction (i) before and (ii) after treatment with DMSO.

CONCLUSIONS

HPGPC on Separon HEMA-S is a suitable method for the determination of the molecular-weight distribution of water-soluble polymers (β-1,3-D-glucans) and for the study of its changes under various conditions, such as heat treatment, sterilization of injection solutions and long-term storage. In this way, the degradation of these compounds can be monitored.

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Determination of polymer polydispersity by gel permeation chromatography

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ABSTRACT

A gel permeation chromatographic method for the determination of polymer polydispersity without the need for preliminary calibration with standards is proposed. Only the chromatogram of the sample under study is required, so the method is convenient for polydispersity investigations of new synthetic polymer materials. The universal dependence of the slope of the calibration graph on the distribution coefficient is used. For verification of the method, polystyrene standards were preliminarily mixed in certain proportions so that the polydispersities of these mixtures could be calculated initially, and then chromatograms of these mixtures were obtained for cross-checking. The proposed method was found to give satisfactory determinations of M_z/M_w, *i.e.*, polymer polydispersity.

INTRODUCTION

The polydispersity of a polymer, U, is usually characterized by the ratio of different average molecular weights, *e.g.*, $U_n = M_w/M_n$ or $U_w = M_z/M_w$. The polydispersity, U_i, is related to the average value of the molecular weight of the sample, M_i (i = n or w), and to its dispersion, σ_i^2, as follows[1]:

$$U_i = 1 + (\sigma_i/M_i)^2 \quad (1)$$

The usual method of determining the polydispersity by gel permeation chromatography (GPC) is as follows[1]. First the calibration dependence is obtained, *i.e.*, the relationship between the retention volume, $V_R = V_o + V_pK$, and the molecular weight of the polymer under study is determined, where V_o and V_p are

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volumes of the mobile and stationary phases, respectively, and K is the distribution coefficient. Normally narrow fractions are used to obtain such a relationship (Fig. 1 shows an example of a calibration dependence for the system of polystyrene and silica gel SW-3000 with a pore diameter $2d = 15$ nm). Next the chromatogram of the sample under investigation is obtained and corrected if necessary by elimination of the instrumental broadening. Then, using the calibration the chromatogram is transformed to the weight function of the molecular weight distribution (MWD), $f_w(M)$ and subsequently the moments of the function

$$M_w = \int_0^\infty f_w(M) M \mathrm{d}M$$

$$M_z = (M_w)^{-1} \int_0^\infty f_w(M) M^2 \mathrm{d}M \qquad (2)$$

are calculated and U_w is determined.

The need for preliminary calibration is a disadvantage of this procedure because narrow-MWD fractions (standards) are not generally available.

In this paper we propose a method for the determination of M_z/M_w that does not require any calibration and is based exclusively on the use of the chromatogram of the sample under study.

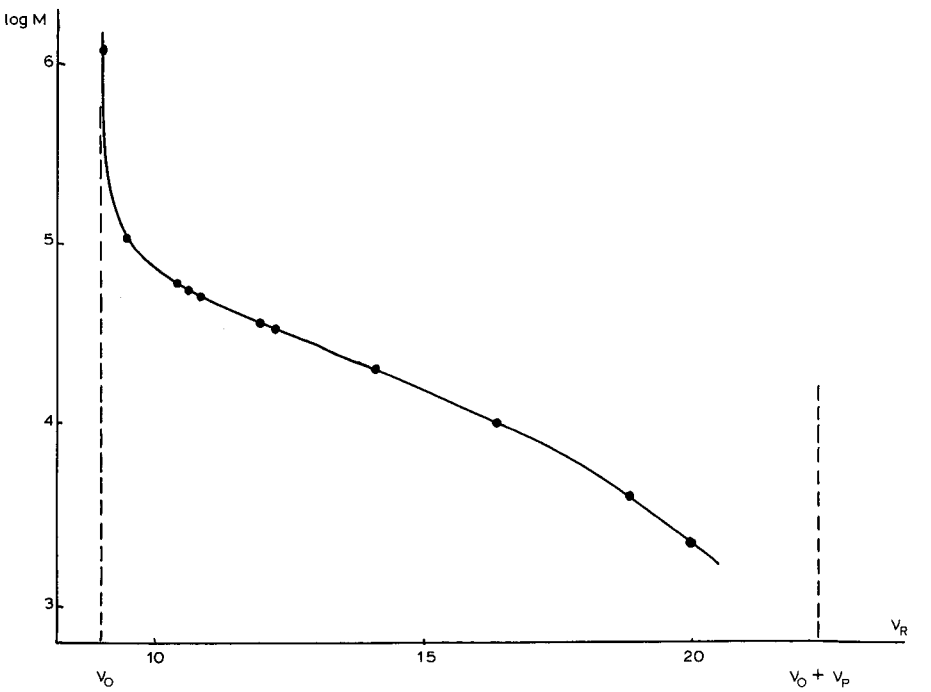

Fig. 1. Calibration dependence for polystyrene and silica gel SW-3000 with pore diameter $2d = 15$ nm, $V_o = 9.0$ ml, $V_o + V_p = 22.45$ ml.

GENERAL THEORY AND INTERNAL STANDARD IN GPC

First we shall show that it is not necessary to obtain a calibration graph of V_R *vs.* ln M in order to determine U_w, it being sufficient just to know the slope of the calibration graph:

$$\psi(K) = \frac{1}{V_p} \cdot \frac{\partial V_R}{\partial \ln M} = \frac{\partial K}{\partial \ln M} \tag{3}$$

Let σ_v be the width of the chromatogram, and assume that the calibration graph is approximately linear within σ_v. It follows that

$$\sigma_v = \left| \frac{dV_R}{d \ln M} \right| \sigma_{\ln M} \approx V_p \cdot \frac{\sigma_w}{M_w} \cdot \psi \tag{4}$$

where $\sigma_{\ln M}$ is the width of the molecular weight logarithmic distribution function. It follows from eqn. 4 that

$$U_w = 1 + (\sigma_w/M_w)^2 \approx 1 + \left(\frac{\sigma_v}{\psi V_p} \right)^2 \tag{5}$$

Hence to determine U_w it is sufficient to know: (a) the chromatographic column characteristics, *i.e.*, the total pore volume, V_p, and the dead volume, V_o; (b) the first and the second moments of the chromatogram, *i.e.*, the distribution coefficient K, and the width of the chromatogram of the sample under study, σ_v; and (c) the slope of the calibration graph, $\psi(K)$, which corresponds to this value of K.

We shall now show that the value of ψ is determined by the value of K only and is independent of both the type of polymer and the type of adsorbent involved.

Let us consider the behaviour of the function $\psi = \partial K/\partial \ln M$, which is inversely proportional to the slope of the calibration graph. Fig. 2 shows normalized calibration graphs in the coordinates log M *versus* K for polystyrene standards and porous silica gel SW-3000, and for narrow disperse dextrans and porous glasses (with different pore diameters). The calibration graphs are similar for these different polymers and adsorbents.

The value of $\psi(K)$ first increases from $\psi(0) = 0$ to a certain value and then decreases to $\psi(1) = 0$. The distribution coefficient for a flexible-chain macromolecule under GPC conditions is known[2,3] to be determined by the ratio of the chain size (average radius of gyration, R) to the pore radius, d. The theory of GPC[2] based on the model of a Gaussian chain in a slit-like pore shows that

$$K \approx \begin{cases} 1 - \dfrac{2}{\sqrt{\pi}} \cdot g, & \text{if } R < d \\ \dfrac{8}{\pi^2} \exp\left[-\left(\dfrac{\pi}{2} \cdot g \right)^2 \right], & \text{if } R > d \end{cases} \tag{6}$$

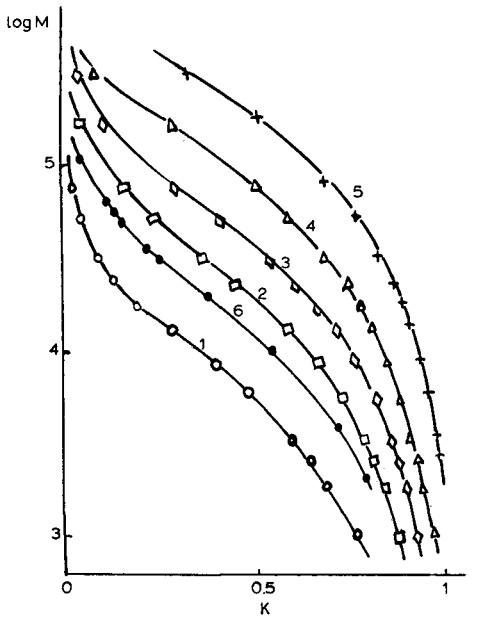

Fig. 2. Normalized calibration dependences for narrow disperse dextrans and porous glasses with pore diameter $2d$ = (1) 8.4, (2) 15.9, (3) 22.7, (4) 31.4 and (5) 51.7 nm[4] and for polystyrene standards and modified silica gel SW-3000 with $2d$ = 15 nm.

where $g = R/d$. For Gaussian chains the relationship

$$\psi = \frac{\partial K}{\partial \ln M} = \frac{g}{2} \cdot \frac{\partial K}{\partial g} \tag{7}$$

is valid, and therefore from eqns. 6 and 7 it follows[3] that

$$|\psi| \approx \begin{cases} (1 - K)/2, & \text{if } K > 0.5 \\ K \ln\left(\dfrac{8}{\pi^2 K}\right), & \text{if } K < 0.5 \end{cases} \tag{8}$$

An analysis proves[3] that eqn. 8 remains valid for pores of various shapes. Hence the dependence $\psi(K)$ is universal for any monodisperse sorbent.

Fig. 3 shows dependence $\psi(K)$ calculated by use of eqn. 8 (solid line). It shows also the experimental data[4] for dextran molecules and porous glasses of various pore diameters and the data for polystyrene and silica gel SW-3000, all these data being obtained by numerical differentiation of the calibration graphs in Fig. 2. It can be seen that universal dependence of the slope of the calibration graph on the distribution coefficient really does exist for various polymers and adsorbents. We shall call this universal dependence an "internal standard" in the GPC of polymers. Experimental corroboration of such a "standard" is shown in Fig. 3.

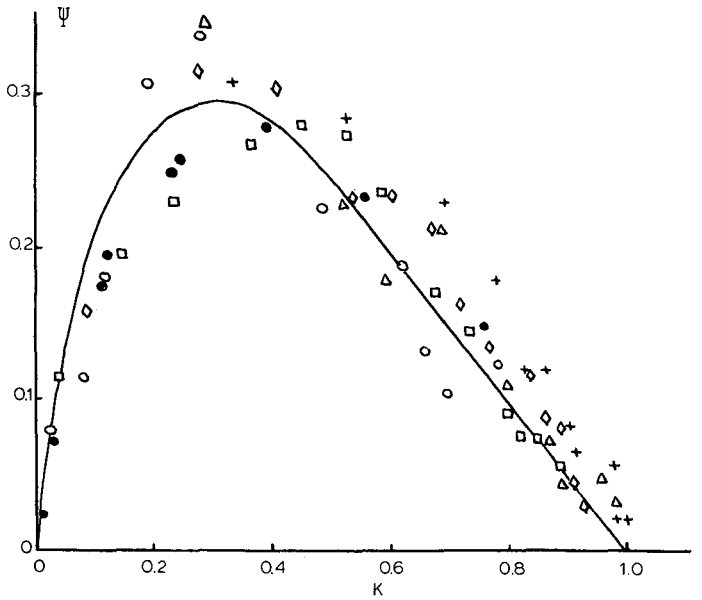

Fig. 3. Slope of calibration graph, $\psi = \partial K/\partial \ln M$, as a function of the distribution coefficient, K, the dependence being obtained by numerical differentiation of the curves in Fig. 2. Symbols as in Fig. 2. The solid line shows the theoretical dependence (eqn. 8).

Hence the procedure for the determination of polydispersity by the proposed method consists in the following: from the value of the distribution coefficient of the sample under study (the first moment of the chromatogram), the value of $\psi = \psi(K)$ is calculated by means of eqn. 8. Subsequently, using both the $\psi(K)$ and the measured value of the chromatogram width σ_v (the second moment), the ratio M_z/M_w is calculated by means of eqn. 5.

EXPERIMENTAL VERIFICATION OF THE METHOD FOR DETERMINATION OF POLYMER POLYDISPERSITY

The proposed method was tested using a Model Trirotar SR2 high-performance liquid chromatograph (Jasco, Tokyo, Japan) with UV detection at 254 nm. A 600 × 7.5 mm I.D. column (LKB, Bromma, Sweden) packed with SW-3000 sorbent (Toyo Soda, Tokyo, Japan) was used with tetrahydrofuran as the eluent. The sorbent is a porous modified silica gel with a particle size of 10 μm and a pore diameter of ca. 15 nm. Polystyrene standards with $M = 2 \cdot 10^3$–$1.11 \cdot 10^5$ (Waters Assoc., Milford, MA, U.S.A.) were investigated. The sample volume (sampling loop) was 20 μl, the polymer concentration in the sample was 1–2 mg/ml, the elution rate was 1 ml/min and all experiments were carried out at ambient temperature (*ca.* 22°C). The quasi-equilibrium character of the process under study and the exclusion mode of the chromatography were verified by checking the constancy of the retention volumes when the elution rate was varied from 0.3 to 2 ml/min and temperature from 10°C to 50°C. The efficiency of the column was *ca.* 12 000 theoretical plates for toluene.

TABLE I

COMPOSITIONS AND MOLECULAR AND CHROMATOGRAPHIC CHARACTERISTICS OF THE MODEL MIXTURES OF POLYSTYRENE STANDARDS

Mixture No.	*Weight-average molecular weights of polystyrene standards, $M \cdot 10^{-3}$*										*Characteristics calculated on the basis of mixture composition*			*Characteristics of samples (mixtures) under study measured experimentally*			
	2.4	*4*	*10*	*20.8*	*34*	*36*	*51*	*54*	*60*	*111*							
	Distribution coefficients of polystyrene standards, K_i																
	—	*0.80*	*0.55*	*0.40*	*0.245*	*0.227*	*0.152*	*0.123*	*0.111*	*0.037*	M_z	M_w	$\frac{M_z}{M_w}$	K	σ_v	$\psi(K)$	$\frac{M_z}{M_w}$
	Mixture composition (%, v/v)																
1							40	40	20		54.2	52.1	1.04	0.14	0.65	0.25	1.04
2								20	30	50	93	84.5	1.10	0.09	0.766	0.20	1.08
3					20	20	20	20	10	10	61.1	52.2	1.17	0.16	0.964	0.262	1.075
4			20	20	10	10	10	10	10	10	61.3	40.6	1.51	0.29	2.49	0.298	1.38
5	10	10	10	10	10	10	10	10	10	10	64	38	1.67	0.35	3.60	0.294	1.8

Correction for the instrumental broadening was performed with the reversed-flow method (Tung's method[5]) as follows. At the moment when the retention volume reached half its maximum value the column was reversed, causing the direction of flow to be reversed. This resulted in the width of the chromatogram being independent of the sample MWD and to be dependent on the instrumental broadening only. For all the samples studied the instrumental broadening was within the range $(\sigma_v)_{approx.} \approx$ 0.19–0.22 ml. The distribution coefficients were calculated as follows:

$$K = (V_R - V_o)/V_p$$

A dead volume of $V_o = 9.0$ ml and a pore volume of $V_p = 13.45$ ml were obtained on the basis of the retention volumes of polystyrene with $M = 1.5 \cdot 10^6$ and toluene. All retention volumes were calculated as the first moments of the corresponding chromatograms.

An investigation of samples with exactly known polydispersities was conducted. These samples were prepared by mixing polystyrene standards in certain preliminarily chosen proportions. The characteristics of the standards and the mixtures prepared are given in Table I. Fig. 4 shows the chromatograms of some mixtures.

The experimental values of the distribution coefficient, K, and the chromatogram widths, σ_v, for all the mixtures investigated are also given in Table I, together with the

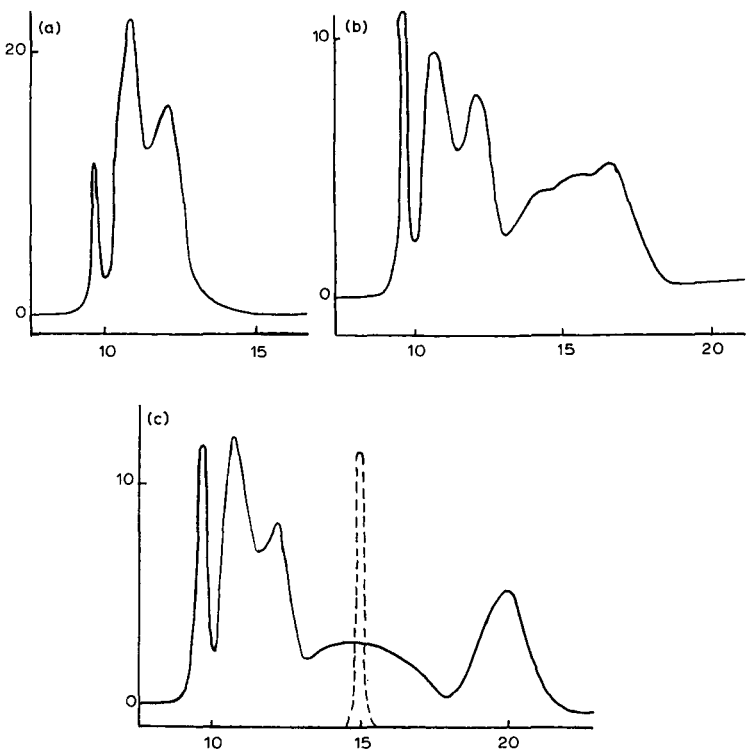

Fig. 4. Experimental chromatograms of mixtures (a) 2, (b) 4 and (c) 5 in Table I. Dashed line shows the instrumental broadening measured by Tung's method[5].

values of $\psi(K)$ determined by means of eqn. 1 and the values of $(M_z/M_w)_{exp}$ calculated by means of eqn. 2.

As can be seen from Table I, the polydispersities obtained experimentally from chromatograms are close to the precise values of M_z/M_w which were calculated previously for all the samples involved.

It should be noted that the proposed procedure requires no preliminary information about the polymer under study.

When verifying the method experimentally, mixtures with a wide range of MWDs were prepared and the chromatograms were of complex shape (see Fig. 4). It is concluded that the method will be useful in both laboratory and industrial practice for the rapid analysis of new synthetic polymer materials.

DISCUSSION

The basis of the proposed method is the concept of internal calibration, *i.e.*, the universal dependence $\psi = \psi(k)$. Eqn. 8, which describes this dependence, follows directly from Casassa and Tagami's theory[2], which is rigorously valid for thermodynamically ideal solvents (so called θ-solvents) with $\nu_\theta = 1/2(\overline{R^2} = CM^{2\nu})$. In practice, good solvents with values of ν between 0.5 and 0.6 are used. In this case

$$\psi = \frac{dK}{d \ln M} = \frac{\partial}{\partial_\theta}\psi_\theta = 2\nu\psi_\theta \tag{9}$$

An important assumption in the derivation of eqns. 8 was monodispersity of the adsorbent, *i.e.*, all the pores are of equal width. With adsorbents with wide pore-size distribution, eqns. 8 can be considered to be approximate. If greater accuracy is to be achieved the experimental dependence $\psi(K)$ should be obtained previously using polymer standards (*e.g.*, polystyrene standards).

It follows from eqns. 8 that ψ reaches its maximum value $\psi_{max} \approx 0.3$ at $K \approx 0.3$. For sorbents with slit-like pores this corresponds to $R^* \approx 2d/\pi$. It is useful to change from consideration of the average pore diameter $2d$ to the specific surface area, $\Sigma = d^{-1}$, which is equal to the ratio of the area of all the pores, S_p, to the total volume, V_p. Then

$$R^* \approx \frac{2}{\pi}(\Sigma)^{-1} \approx 0.64 V_p/S_p \tag{10}$$

Eqn. 10 determines the dependence of the point of inflection of the calibration graph on the pore sorbent characteristics and allows the pore specific surface area to be obtained. The experimental data are described[6] by the empirical relationship $R^* \approx 0.7(\Sigma)^{-1}$, and this is in good accordance with eqn. 10.

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CHROM. 22 387

Note

Gel permeation chromatography for the determination of the molecular mass distribution of some industrial proteins

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Gel permeation chromatography (GPC) is an important method of purification and is used in the analysis of proteins, nucleic acids and other bioligands and biopolymers with gels of different pore sizes as the stationary phase[1–3].

EXPERIMENTAL

Gel preparation

Sephadex gels which are dextran polymers cross-linked by epichlorohydrin, were purified by decantation and swollen in the solvent used for elution. Homogeneity of the gel formed in the column limits the column resolution. Therefore, the swollen gel is introduced into the column in the form of a suspension.

Gel filtration

Table I gives the properties of model proteins used for preparation of the calibration graphs. The proteins were filtered in a series of glass columns (45 × 1.5 cm, 55 × 1.5 cm and 55 × 2.0 cm I.D.) filled with Sephadex G-100 grains of size 40–120 μm (Pharmacia, Uppsala, Sweden). Degassed phosphate buffer (Na_2HPO_4 + KH_2PO_4) of concentration 0.066 mol/l and pH 6.6 was used as the eluent. Elution was carried out without a pump at a rate of about 12 ml/h and at an appropriately selected

TABLE I

PROPERTIES OF MODEL PROTEINS USED IN THE CALIBRATION OF THE GEL FILTRATION MOLECULAR MASS DISTRIBUTION ANALYSIS

Model protein	*Molecular mass*	*Isoelectric point*	*UV–VIS diagnostic band (nm)*
γ-Globulin	180 000	5.6–8.2	280
Serum albumin	69 000	4.7–4.9	280
α-Amylase	50 000	7.0	280
Egg albumin	42 000	4.6	280
Trypsin	23 000	10.9	280
Cytochrome *c*	13 000	9.8–10.1	412

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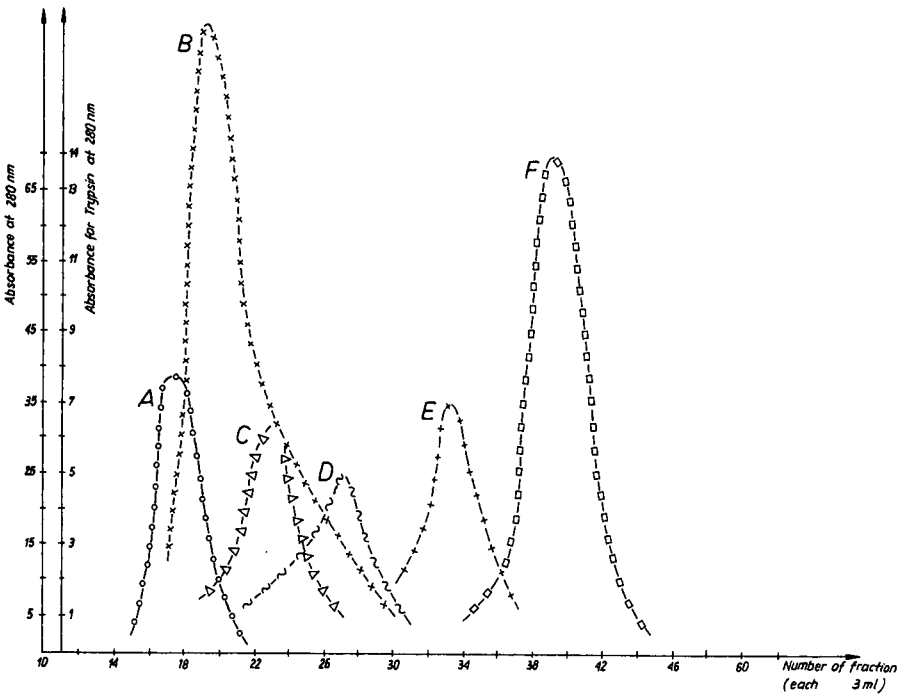

Fig. 1. Elution curves for several model proteins and blue dextran obtained from a 55 × 1.5 cm I.D. Sephadex G-100 column. A = Blue dextran; B = γ-globulin; C = serum albumin; D = egg-albumin; E = trypsin; F = Cytochrome *c*.

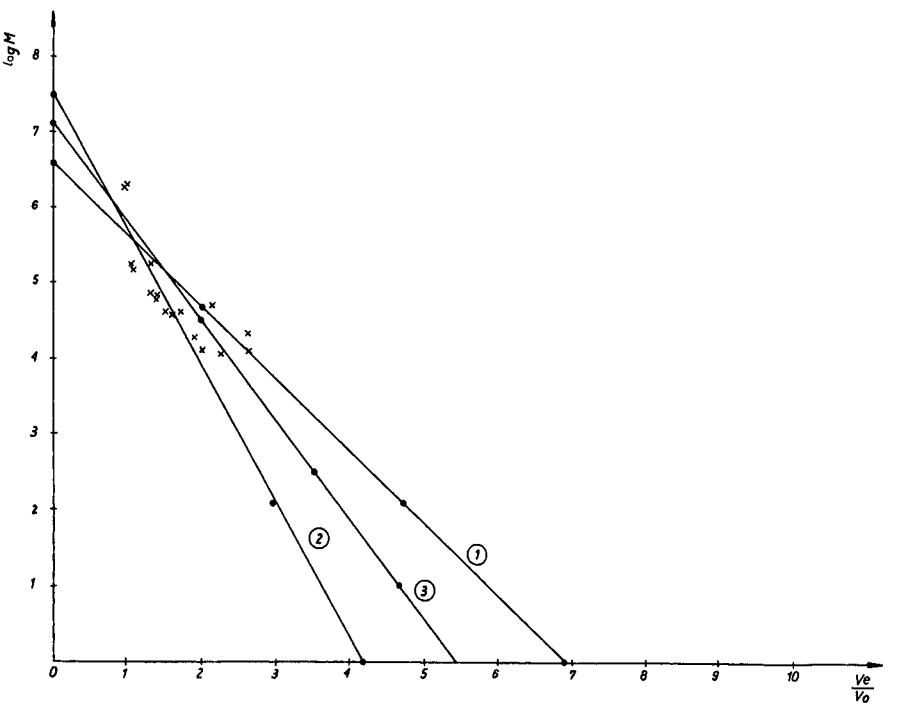

Fig. 2. Calibration graphs of log M *vs.* V_e/V_0 for gel filtration of proteins using Sephadex G-100 columns: 1 = 45 × 1.5 cm I.D.; 2 = 55 × 1.5 cm I.D.;3 = 55 × 2.0 cm I.D.

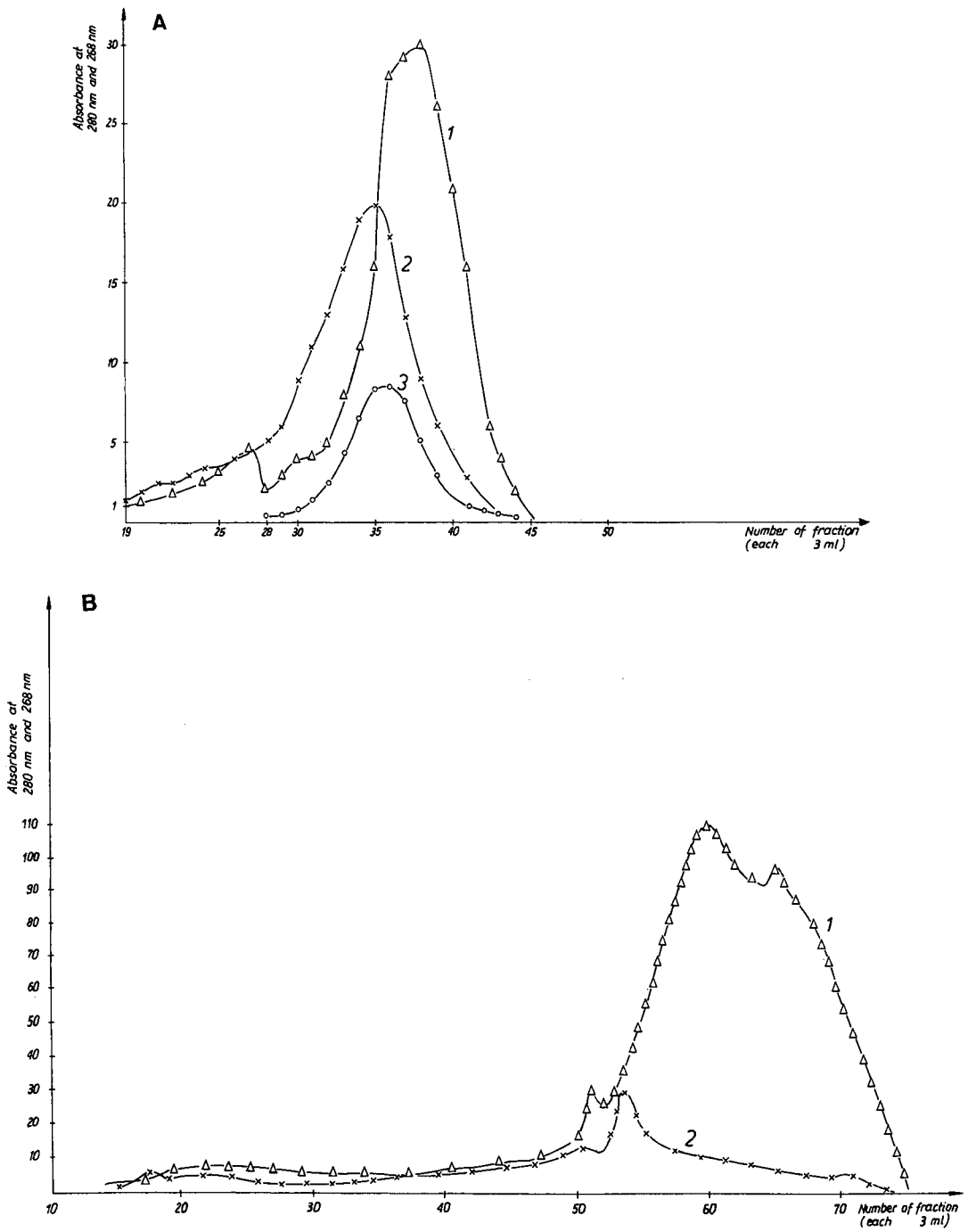

Fig. 3. (A) Elution curves of raw, α-amylase sample and ultrafiltration retentate and filtrate. 1 = α-Amylase sample; 2 = retentate; 3 = filtrate. Column: 55 × 1.5 cm I.D. (B) Elution curves of raw pectase sample and ultrafiltration retentate. 1 = Pectase sample; 2 = retentate. Column: 55 × 1.5 cm I.D.

hydrostatic pressure. The void volume of the column (V_0) was determined by means of blue dextran ($M = 2 \cdot 10^6$). The course of the separation was monitored by recording a UV–VIS spectrum for every fraction (3 ml) using a Specord UV–VIS spectrometer (Carl Zeiss, Jena, G.D.R.). Elution curves of several model proteins and blue dextran are shown in Fig. 1.

Determination of calibration graph

Determination of a calibration graph of log M *vs.* V_e/V_0 is essential for calculating the molecular mass distribution of biopolymers. Directional coefficients (a and b) are calculated from the log $M = a(V_e/V_0) + b$ relationship using the least-squares method. The elution volume (V_e) for reference proteins was determined on the basis of the results obtained during gel permeation on Sephadex G-100. Fig. 2 shows the calibration graphs for the gel permeation of proteins.

RESULTS

Gel permeation was performed for a series of samples of α-amylase, several pectinases (industrial samples of bacterial origin) and streams of their filtrates and concentrates obtained from ultrafiltration on polyacrylonitrile ad poly(vinyl chloride) membranes. The elution curves are shown in Fig. 3. A knowledge of the molecular mass distribution for the filtrate and concentrate makes it possible to characterize the ultrafiltration process compared with the molecular mass distribution of the original sample of the industrial active protein, *e.g.*, α-amylase or pectinase. Molecular mass distributions of these samples obtained by gel chromatography are given in Figs. 4 and 5.

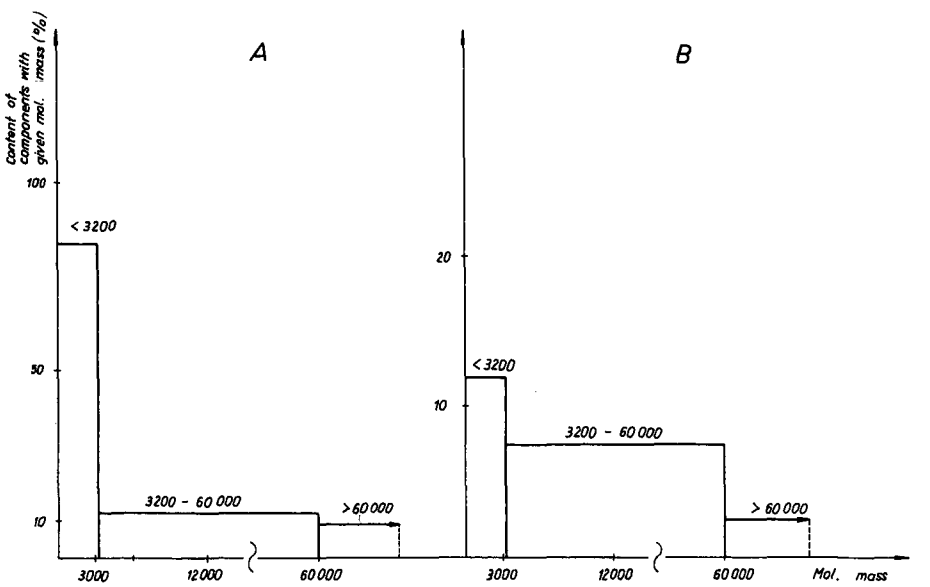

Fig. 4. Gel chromatographic molecular mass distributions of (A) raw pectase sample and (B) ultrafiltration retentate.

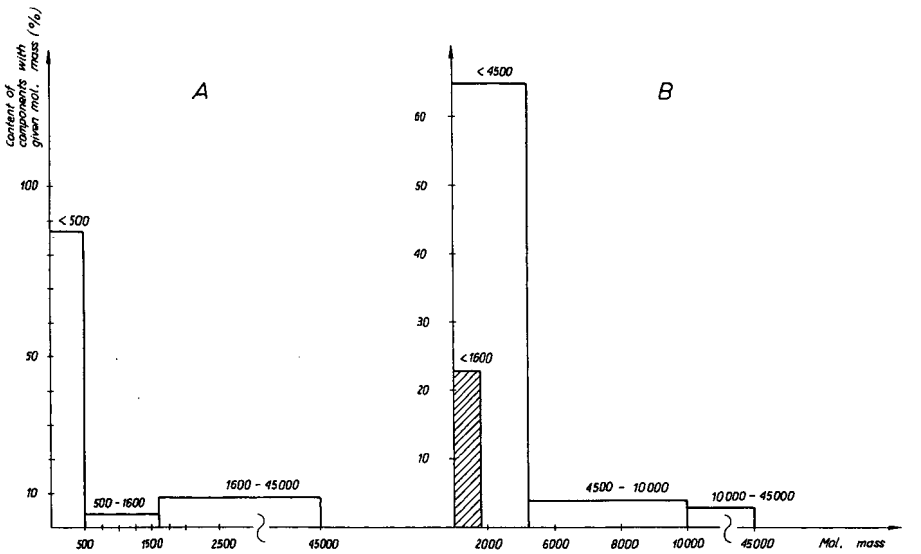

Fig. 5. Gel chromatographic molecular mass distributions of (A) raw α-amylase sample and (B) ultrafiltration retentate (open boxes) and filtrate (hatched box).

CONCLUSION

The proposed method allows the determination of the molecular mass distribution of protein mixtures in the streams emanating from ultrafiltration of technical proteins using polyacrylonitrile and poly(vinyl chloride) membranes.

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Phase transition chromatography of polyesters on macroporous glycidyl methacrylate–ethylene dimethacrylate copolymers

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ABSTRACT

The chromatographic behaviour of oligoesters of phenolphthalein and terephthalic acid in columns packed with a polymeric sorbent based on the macroporous strongly cross-linked copolymer glycidyl methacrylate–ethylene dimethacrylate was studied. The possibility of the effective separation of oligomers according to the type of terminal groups was demonstrated. For comparison the sorbent Silasorb 600 was used.

INTRODUCTION

During the last decade, methods for the separation of oligomers according to the type of terminal groups (functionality) have been developed[1]. It has been demonstrated[2] that the shift from size-exclusion chromatography to the adsorption mode by changing the adsorption energy, of the polymer unit by decreasing the eluent polarity is connected with the phase transition of the polymer. At this boundary, named the "critical region", the size separation of linear chains disappears and separation according to structural heterogeneity, particularly its functionality, can be achieved. The critical regions have been found for a number of polymers and sorbents.

Hydrolysed macroporous glycidyl methacrylate–ethylenedimethacrylate (GMA–EDMA) copolymers have been used previously[3] as universal sorbents for the high-performance gel permeation chromatography (GPC) of polymers with both aqueous and weakly polar organic mobile phases.

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In this work, the posssibility of separating aromatic oligoesters according to the types of terminal groups on GMA–EDMA copolymers was studied.

EXPERIMENTAL

The functionality types of oligoesters of terephthalic acid and phenolphthalein were investigated, where the functionality $f = 0, 1, 2$ represents 0, 1 and 2 terminal phenolic hydroxy groups per molecule, respectively.

The samples were prepared by high-temperature or heterophase[4] polycondensation and characterized[5] by osmometry, GPC and high-performance liquid chromatography. The molecular weights and functionality values of the oligoesters are given in Table I.

TABLE I

NUMBER- ($\bar{M}_n$) AND WEIGHT-AVERAGE ($\bar{M}_w$) MOLECULAR WEIGHTS AND NUMBER-AVERAGE FUNCTIONALITY VALUES ($\bar{f}_n$) OF OLIGOESTERS

Sample	$\bar{M}_n$	$\bar{M}_w$	$\bar{f}_n$
1	1770	2060	1.30
2	2240	3790	0.90
3	1750	2080	1.95
4	1090	1140	0.00

Chromatographic experiments were carried out using a Milichrom 1 apparatus (Scientific Instruments, Orel, U.S.S.R.). Columns of 62 × 2 mm I.D. and 280 × 2 mm I.D. were packed with polymeric sorbents based on hydrolysed macroporous strongly cross-linked GME–EDMA copolymers (G5 and 6/11H). Mixtures of hexane with tetrahydrofuran (THF) or methylene chloride were used as the mobile phase.

The results were compared with those obtained with a 62 × 2 mm I.D. silica column (Silasorb 600; Lachema, Brno, Czechoslovakia).

RESULTS AND DISCUSSION

Fig. 1 shows the transition from the size-exclusion separation mode to the adsorption mode on (a) 6/11H and (b) G5 sorbents using THF/hexane mixtures. The critical region for these sorbents was found at 30% (v/v) of hexane (6/11H) and between 10 and 20% of hexane (G5) in the mixture. The distribution coefficients (K_d) of bifunctional molecules [$K_d^{(2)}$] under these conditions were found to be too small [$K_d(2) = 1.3$ for 6/11H and $1.23 < K_d(2) < 2$ for G5] for separation according to functionality.

A similar shift from the size-exclusion to the adsorption mode was observed[5] on the Silasorb 600 column. The critical region corresponds to a hexane content equal of 30% in the THF–hexane mixture, and $K_d(2) = 1.5$. Hence the oligoesters cannot be separated according to functionality in THF-containing mobile phases.

Fig. 2 shows the shift in chromatographic mode at the critical region for GMA–EDMA sorbents using methylene chloride–hexane mixtures. The critical

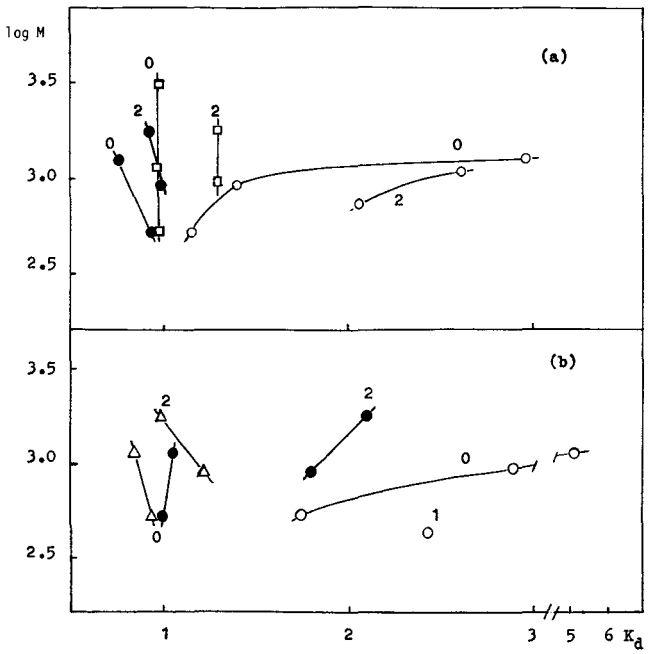

Fig. 1. Transition from the exclusion separation mode to the adsorption mode on (a) 6/11H and (b) G5 sorbents using THF–hexane mixtures. The numbers on the curves correspond to the functionality values. Hexane content in mobile phase: △ = 10; ● = 20; ○ = 30; □ = 40%.

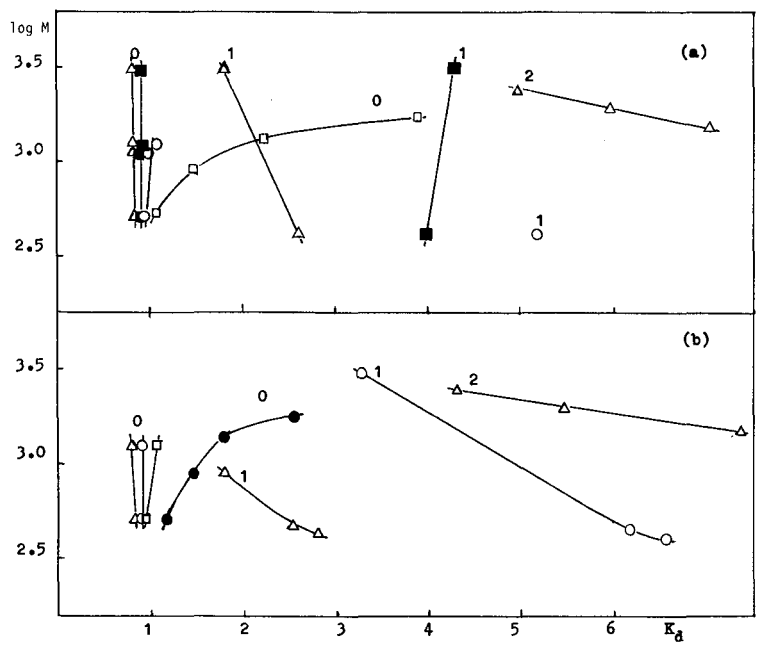

Fig. 2. Transition from the exclusion separation mode to the adsorption mode on (a) 6/11H and (b) G5 sorbents using methylene chloride–hexane mixtures. The numbers on the curves correspond to the functionality values. Hexane content in mobile phase: △ = 0; ■ = 20; ○ = 30; □ = 40; ● = 50%.

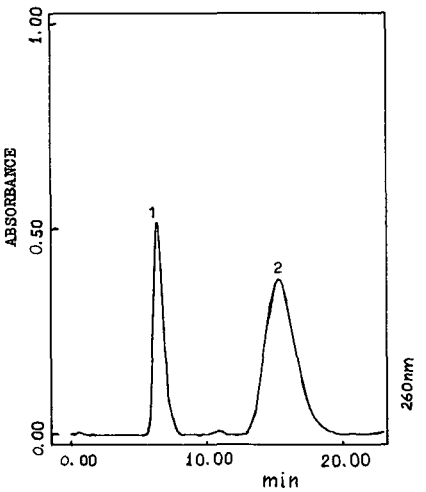

Fig. 3. Chromatogram of sample 1 obtained in the critical region on the 6/11H column. Eluent: methylene chloride–hexane (70:30); flow-rate, 100 μl/min. Peaks: 1 = non-functional fraction; 2 = monofunctional fraction.

region corresponds to a 20% content of hexane in the mixture for 6/11H and between 30 and 40% of hexane for G5. The retention times of functional molecules are very large in these mobile phases [$K_d(1) = 4$ for 6/11H and $K_d(1) > 10$ for G5]. The bifunctional molecules proved to be irreversably adsorbed on G5.

Fig. 3 shows a chromatogram obtained in the critical region on the 6/11H column. The two peaks correspond to non-functional and monofunctional fractions of oligoesters.

The results were compared with those obtained on a Silasorb 600 column using methylene chloride-containing mobile phases. In pure methylene chloride, separation of oligoesters by adsorption occurs. A stronger eluent, *e.g.*, 1–2% of 2-propanol, must be added in order to reach the critical region, as illustrated in Fig. 4. In the critical region irreversible adsorption of bifunctional molecules was observed on both the silica and organic sorbents.

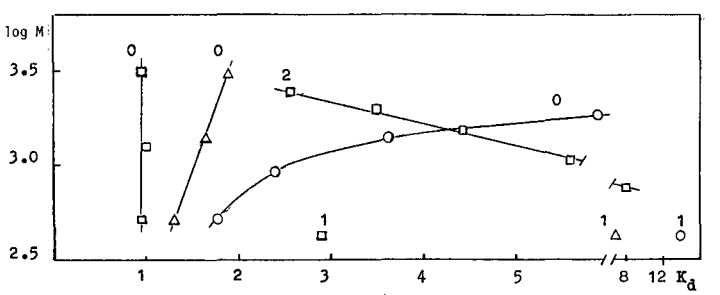

Fig. 4. Transition from the exclusion separation mode to the adsorption mode on Silasorb 600 using methylene chloride–2-propanol mixtures. 2-Propanol content in mobile phase: ○ = 0.5; △ = 1; □ = 2%.

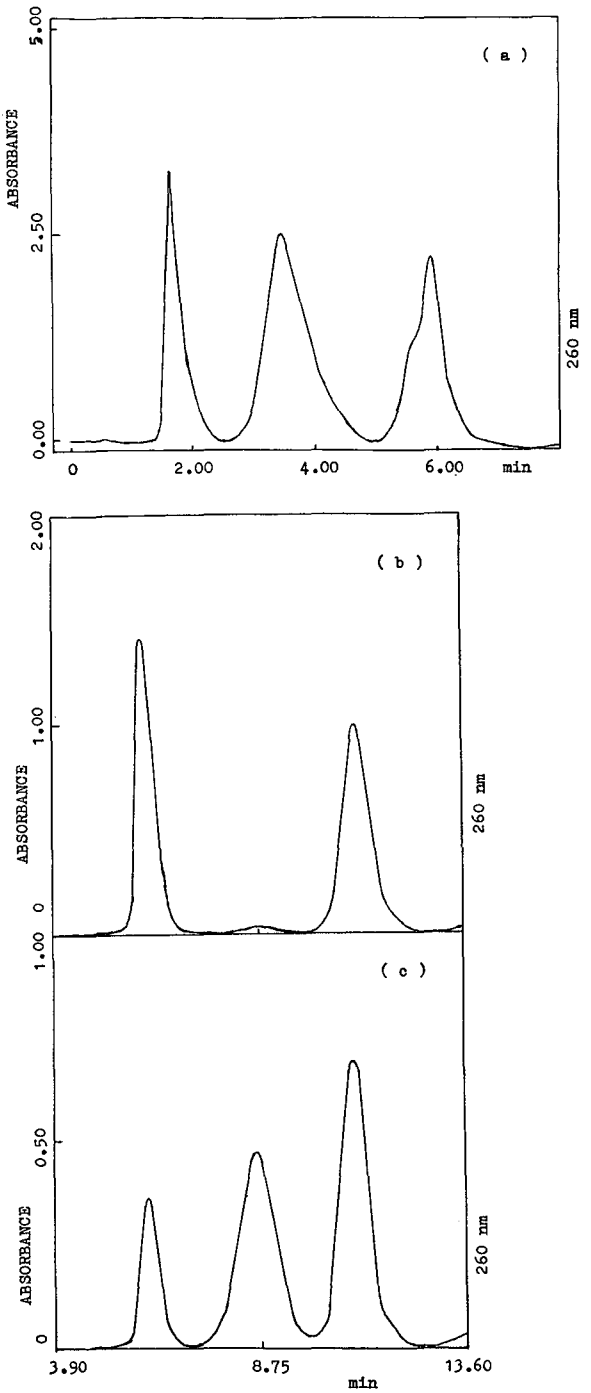

Fig. 5. Chromatograms of samples (c) 1 and (a) 2 and (b) a mixture of samples 3 and 4, obtained by the gradient elution technique on (a) the G5 column and (b and c) the 6/11H column. Mobile phases: (a) step 1 (up to 4 min); methylene chloride–hexane (60:40); step 2 (up to 8 min); methylene chloride–hexane–THF (30:20:50); step 3 (up to 12 min); THF; (b and c) step 1 (up to 6 min): methylene chloride–hexane (70:30), step 2 (up to 15 min): methylene chloride–hexane–THF (47:20:33). Flow-rate is 100 μl/min.

From the comparison of the two types of sorbent the following conclusions can be drawn. These sorbents contain different functional groups on the the surface, *i.e.*, silanol groups on silica and hydroxy groups on organic sorbents. In a solvent such as methylene chloride, which does not show specific interactions with the sorbent and sorbate, the adsorption energy of the polymer unit is different for each sorbent. The energy reaches a critical value in solvents of different elution strengths calculated according to Snyder's theory[6] for alumina ($0.62 < \varepsilon < 0.66$ on Silasorb 600 and $\varepsilon = 0.40$ on 6/11H).

In contrast, the critical regions both for sorbents in THF-containing solvents approximately coincide ($\varepsilon = 0.43$). This means that specific interactions of the solvent with both the sorbate and sorbent cause a decrease in the adsorption energy of the polymer unit on the silica surface to the same extent as on hydroxy groups and simultaneously decreases the difference between the adsorption energies of a middle unit and the terminal group.

The results of this work show that the chromatography in the critical region must be modified for the effective separation of the studied oligoesters by the gradient elution technique. A concave profile of the gradient was obtained with a stepwise change in the eluent composition. The first stage of elution was accomplished with the critical mixture of methylene chloride and hexane and then, after elution of the non-functional fraction peak, the composition of the eluent was changed by addition of THF (two or three stages). Examples of such chromatograms are shown in Fig. 5. Samples 1 and 2 consisted of three fractions of different functionality (Fig. 5c and a). Fig. 5b shows for comparison the chromatograms of a mixture of the non-functional sample 4 and the bifunctional sample 3.

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Increasing the purity of polyuronic sorbents

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ABSTRACT

The method of elution ion-exchange chromatography was applied to increase the purity of polyuronic sorbents (apple, carrot and sunflower pectins). The columns is packed with the adsorbent to be purified. Organic solvents *e.g.*, methanol, ethanol and acetone containing HCl are used for the elution at 20, 40 and 50°C. Elimination of mineral substances and nitrogen-containing compounds and an increase in the polyuronic acid content were observed. It was found that efficient purification of pectic substances is possible under the adopted conditions. Partial de-esterification was observed with increase in the pectin purity and the reaction speed was increased by increasing the temperature. Protein–peptide admixtures removed from these pectins, the greatest decrease in the content of nitrogenous substances being observed with carrot pectin.

INTRODUCTION

Pectic substances, pectic acid and alginic acid are polyuronides with well expressed adsorption and ion-exchange properties. Their ability to interact with amines[1], amino acids[2], alkaloids[3], phenols[4], steroids[5], metal ions[6,7], etc., is well known and modified polyuronides have found application in affinity chromatography[8].

Our studies have indicated that polyuronides can be successfully applied as optically active sorbents for the chromatographic resolution of racemic bases[9]. Their good ion-exchange properties make them suitable detoxicants in cases of heavy metal poisoning[5,10] and also as stabilizers of alcoholic drinks against cation turbidity[11].

The good adsorption and ion-exchange properties of polyuronides are also the reason why they retain considerable amounts of admixtures of mineral and organic origin in the process for their production. Therefore, polyuronides are produced with a relatively low purity. However, the requirements for the purity of polyuronides intended for medical and chromatographic purposes have increased recently.

Different methods have been suggested for increasing the purity of pectic substances, *e.g.*, passing dilute pectic solutions through columns with cation exchangers to remove pigments and metal ions[12,13] or static treatment with mineral acids

in an aqueous–alcoholic medium[14–17]. The multiple dissolution of pectin in water and reprecipitation with ethanol acided with HCl has also been applied for purification on the laboratory scale. This method, however, is ineffective and tedious. The low efficiency of static methods is caused by the stability of the salt-like compounds formed from pectins (*e.g.*, pectic acids) and by impurities having a positive electrical charge (polyvalent cations, peptides, amino acids, amines, etc.). The dissociation of such complex salts is possible by column chromatography[9], and we have carried out experiments to increase the purity of pectic preparations by elution of a column with organic solvents containing dilute hydrochloric acid.

EXPERIMENTAL

Materials and reagents

Commercial apple, sunflower and carrot pectins were used. The organic solvents (methanol, ethanol, acetone) were of analytical-reagent grade.

Experimental set-up

The experiments for purification of pectin were carried out as follows. The pectic preparation was hydrated by swelling for 1 h in a mixture of water and the organic solvent (1.5:1) and a chromatographic column (500 mm × 14 mm I.D.) was packed with the material obtained. The pectic preparation acted as an adsorbent. A water-diluted organic solvent containing hydrochloric acid (2.2% and 3.1% HCl) was used as the eluent. In this way the hydrogen ions of the eluent displaced the metal or organic cations bonded with the carboxyl groups of the pectic macromolecules. Moreover, the acidic medium increased the solubility of other organic impurities in organic solvents. After elution, the pectic preparation was removed from the column, mixed with a five times greater volume of 70% ethanol and washed until the washings were neutral.

The purification of pectin by dissolution and precipitating was performed as follows (Table I, experiment 2). A 10-g amount of initial pectin was dissolved in 1000 ml of distilled water and an equal volume of 95% ethanol containing 2.2% HCl was added. The precipitated pectin was filtered, washed until the washings were neutral and dried. The pectin obtained was precipitated twice more in the same way and the pectin dried after the third precipitation was subjected to analysis.

Methods of analysis

The degree of esterification and the purity of pectic preparations were determined by the neutralization method with phenol red as the indicator[17].

The amino acid content of the pectic preparations was determined as follows. A 0.5-g amount of a pectic preparation were mixed with 10 ml of 3 *M* sulphuric acid and heated at 108°C (sulphuric acid boiling point) for 40 h. The acidic hydrolysate was neutralized with calcium hydroxide, the precipitate was filtered and the clear filtrate was subjected to amino acid analysis on an HD-1200 E automatic amino acid analyser (Zavod SNP n.p. Žiar nad HRONOM, Czechoslovakia).

The mineral content of the purified pectins was determined by atomic absorption spectrometry. The ash from each sample was dissolved in 4 ml of concentrated HCl and diluted to 25 ml with demineralized water. These solutions were analysed on a Perkin-Elmer Model 4000 atomic absorption spectrometer.

RESULTS AND DISCUSSION

Initial experiments on the purification of apple pectin by elution in a column with ethanol–hydrochloric acid (2.2% HCl in 40% ethanol) were carried out for 48 h at room temperature. Parallel experiments were performed by dissolving the apple pectin three times in water and reprecipitating it in ethanol–hydrochloric acid. The results obtained are given in Table I.

The results show that both methods of purification give a substantial increase in purity, from 58 to 72–74%, the eluent method being slightly more efficient. Moreover, it should be noted that the removal of the mineral components and nitrogen substances with the elution method is considerably more complete.

It should also be noted that under these conditions a process of de-esterification takes place, which undoubtedly depends on the duration of the acid treatment and is, therefore, more clearly expressed with the eluent method for purification (experiment 1).

No substantial differences in the efficiency of purification of the three pectins (apple, carrot and sunflower) were observed between the three solvents, except with regard to the degree of esterification. The smallest changes were observed with methanol, obviously because of the equilibrium of the de-esterification process in the presence of methanol–hydrochloric acid[15,16].

In another series of experiments, the effect of temperature during elution with ethanol–hydrochloric acid was tested. It was found that an increase in temperature from 20 to 50°C speeded up of the pectin purification, de-esterification also being more strongly expressed.

The data in Table I show that the greatest differences between experiments 1 and 2 are in the nitrogen content of the purified pectins, which is more than halved with the eluent method but remains almost unchanged with the dissolution and reprecipitation method. It was therefore of interest to analyse the tested pectic preparations with respect to this parameter. It has been established that the major part of the nitrogenous compounds in apple and citrus fruit pectins are polypeptides and proteins[18–20], whose concentrations vary from 0.5 to 3.5%. For this reason the samples were subjected to

TABLE I

COMPARATIVE DATA FOR APPLE PECTIN PURIFIED AT 20°C BY ELUTION IN A COLUMN AND REPRECIPITATION

Parameter	*Control pectin sample*	*In a column, 48 h (experiment 1)*	*Pectin precipitated three times from ethanol–HCl (experiment 2)*
Degree of esterification (%)	74.3	65.6	69.3
Purity (%)	58.1	74.4	72.6
Ash content (%)	3.16	0.16	0.75
Nitrogen content (Kjeldahl) (%)	0.39	0.16	0.37
Ethanol consumption (ml per 1 g of pectin)	–	250	300

TABLE II

AMINO ACID COMPOSITION (%) OF PECTIN HYDROLYSATES

Hydrolysis for 40 h in 3 *M* sulphuric acid at 108°C.

Amino acid	*Apple pectin*		*Carrot pectin*		*Sunflower pectin*	
	Initial	*Purified after 48 h elution at 20°C*	*Initial*	*Purified after 80 h elution at 20°C*	*Initial*	*Purified after 50 h elution at 20°C*
Lysine	0.036	0.020	0.064	0.014	0.034	0.026
Histidine	–	–	0.016	–	0.014	Trace
Arginine	–	–	0.032	–	–	–
Aspartic acid	–	–	0.024	0.016	Trace	Trace
Threonine + serine	0.084	0.054	0.108	0.048	0.104	0.036
Glutamic acid	0.032	–	0.094	0.012	0.082	0.076
Proline	–	–	Trace	Trace	Trace	Trace
Glycine	0.036	0.026	0.054	0.016	0.056	0.028
Alanine	0.028	0.016	0.062	0.020	0.068	0.036
Cystine	Trace	–	–	–	–	–
Valine	0.048	0.032	0.048	0.026	0.054	0.044
Methionine	Trace	–	–	–	–	–
Isoleucine	0.034	0.016	0.032	0.014	0.040	0.028
Leucine	0.046	0.028	0.108	0.030	0.074	0.054
Tyrosine	–	–	0.018	0.012	0.024	0.012
Phenylanine	0.022	0.014	0.028	0.016	0.036	0.028
Total	0.366	0.206	0.688	0.224	0.586	0.368

acid hydrolysis and their amino acid composition was determined. Table II gives data for apple, carrot and sunflower pectins. The data for the initial pectin samples show that quantitatively and qualitatively carrot pectin is the richest of the three in amino acids.

Table III gives some data for the cation composition of apple pectin subjected to purification by the eluent method. The analysis was performed with four different

TABLE III

MINERAL CONTENT OF APPLE PECTIN PREPARATIONS OBTAINED AFTER PURIFICATION BY THE ELUTION METHOD

Solvent, 2.2% HCl in 40% ethanol; temperature, 40°C.

Sample	*Duration of purification (h)*	*Amount (mg/g)*							
		Na	*K*	*Mg*	*Ca*	*Cu*	*Zn*	*Mn*	*Fe*
Control	0	0.30	2.10	3.19	5.90	0.14	0.03	0.02	0.75
1	4	0.09	0.08	0.02	0.03	0.02	0.01	0.00	0.18
2	8	0.07	0.02	0.02	0.06	0.02	0.01	0.00	0.16
3	12	0.02	0.02	0.01	0.04	0.02	0.01	0.00	0.13
4	48	0.04	0.02	0.01	0.02	0.02	0.01	0.00	0.10

durations of purification. The results show that the removal of a major part of the mineral admixtures takes place during the first 4 h. Iron cations, however, are removed very slowly and even after 48 h of elution their content is about 0.1 mg/g.

CONCLUSIONS

It has been shown that it is possible to purify efficiently pectins from fruit, vegetables and other plants using column elution with ethanol, methanol or acetone containing hydrochloric acid. An increase in temperature speeds up the removal of admixtures.

Partial de-esterification of pectin was observed with the increase in purity. This process is also speeded with an increase in temperature. De-esterification is slowest using elution with methanol.

A difference was established in removing the protein peptide admixtures of apple, carrot and sunflower pectins. The greatest decrease in amino acid content using the elution method was observed with carrot pectin.

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CHROM. 22 200

Ion-exchange high-performance liquid chromatographic separation of peptides with UV photometric and electrochemical detection

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ABSTRACT

The ion-exchange separation of dipeptides and some higher peptides was studied using a Separon HEMA Bio 1000 CM weak cation-exchange column. The effects of the stationary phase properties (particle size, column dimensions) and the mobile phase parameters (flow-rate, composition, pH, organic modifier content) on the separation were studied. Both electrostatic and hydrophobic interactions are involved in the separation; in the series of dipeptides, the retention depends on the hydrophobicity of the amino acid side-chain and its carbon number.

UV photometric (at 210 nm) and electrochemical detection (voltammetric at a copper electrode and tensammetric at a mercury dropping electrode) were tested and their sensitivities compared. UV photometric detection is about ten times more sensitive than electrochemical detection, the sensitivities of amperometric detection with a copper electrode and tensammetric detection being comparable. An advantage of tensammetric detection is that the response is independent of the length of the peptide side-chain; on the other hand, tensammetric detection is very sensitive to the presence of surface-active substances in the mobile phase.

INTRODUCTION

Liquid chromatographic analyses of peptides are important in many fields of science, especially in biochemistry, medicine and biology[1–3]
The most common separation systems are reversed-phase and ion-exchange high-performance liquid chromatography (HPLC) (see, *e.g.*, refs. 3–7); size-exclusion techniques are suitable for group separations of peptides and for analyses of long-chain peptides and proteins[8]. Peptides are usually detected by UV photometry at low wavelengths (*e.g.*, ref. 9), but amperometric detection is also possible, using primarily mercury[10–12] but also carbon[11,13–15] electrodes. Peptides exhibit surface activity and hence it can be assumed that tensammetric detection[16] should also be possible.

Amperometric detection on a passivated copper electrode has been shown[17,18]

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to be advantageous for many substances that rapidly form stable complexes with copper (II) ions and it has been applied to the detection of short peptides separated in a reversed-phase chromatographic system[19]. However, this method of detection imposes severe limitations on the mobile phase composition; the pH must not be lower than 6 and only certain buffers (*e.g.*, phosphate or carbonate) permit sensitive measurement. The separation of peptides in reversed-phase systems is not optimum at these pH values and substantially lower values are preferable[20–22].

Therefore, in this work we decided to test a newly developed[23] column packing material, HEMA Bio 1000 CM (Tessek, Prague, Czechoslovakia), based on a hydroxyethylmethacrylate–ethylene dimethylacrylate copolymer modified with carboxymethyl groups. It is a weak cation exchanger and hence permits the use of mobile phases with a pH higher than the range permissible with the chemically bonded alkyl phases. We studied the conditions for the separation of short peptides on this stationary phase and the possibilities of combining this separation with UV photometric detection, amperometric detection on a copper electrode and tensammetric detection on a mercury electrode.

EXPERIMENTAL

The measurements were carried out on an HPLC instrument consisting of an LC–UV variable-wavelength photometric detector and an LC–XPD pump (both from Pye Unicam, Cambridge, U.K.), a Rheodyne Model 7125 injector with a 20-μl external loop and and ADLC 1 amperometric detector (Laboratorní Přístroje, Prague, Czechoslovakia) with a voltammetric cell of our own construction[24], containing a tubular copper working, silver/silver chloride (Ag/AgCl) reference and a stainless-steel counter electrode. Tensammetric detection was performed with an EDLC detection cell containing a static mercury drop electrode[25] (Laboratorní Přístroje) and an OH-105 a.c. polarograph (Radelkis, Budapest, Hungary). A TZ 4620 dual-line recorder (Laboratorní Přístroje) was used to record simultaneously the photometric and voltammetric signals.

Prior to measurement, the copper electrode was activated in the mobile phase for 15 min at -0.3 V and then measurement itself was carried out at $+0.15$ V. All the potential values are referred to the Ag/AgCl reference electrode. All the measurements were performed at laboratory temperature (20 ± 2°C).

The characteristics of the column used are given in Table I. The stainless-steel columns were products of Tessek.

The mobile phases involved (a) aqueous phosphate buffers containing various concentrations of NaH_2PO_4 and methanol as the organic modifier, (b) an aqueous acetate buffer and (c) a 0.1 *M* aqueous solution of sodium perchlorate. Before use, the mobile phases were degassed in an ultrasonic bath. The mobile phase pH was adjusted by addition of solutions of phosphoric, acetic or perchloric acid and sodium hydroxide.

The test peptides were obtained from Sigma (St. Louis, MO, U.S.A.). All the other chemicals were of analytical-reagent grade (Lachema, Brno, Czechoslovakia) and were used as received.

TABLE I

CHARACTERISTICS OF THE HEMA COLUMNS AND THEIR PERFORMANCE

Parameter	*Column*			
	I	*II*	*III*	*IV*
Dimensions (length × I.D., mm)	80 × 8	80 × 8	250 × 8	250 × 4
Particle size (μm)	7	10	7	10
Plate number[a]:				
a	940	800	1850	835
b	790	650	1560	700
c	750	580	1480	660
Resolution[a, b]:				
a	1.6	1.5	2.7	1.5
b	1.3	1.1	2.2	1.0
c	1.05	1.0	1.75	0.9

[a] Flow-rate: (a) 0.5, (b) 1.0 and (c) 1.5 ml/min.
[b] Gly–Val and Gly–Leu pair.

RESULTS AND DISCUSSION

Separation

The weakly acidic cation exchanger HEMA Bio 1000 CM exhibits both ion-exchange properties and hydrophobic interactions with the mobile phase and hence is particularly well suited for separations of proteins and their fragments. Four columns with different lengths, inside diameters and particle sizes were tested (see Table I). The narrower column exhibits a hydrodynamic resistance four times greater than that of the wider columns and its efficiency is less than half (columns III and IV). The effect of the particle size is not very pronounced (columns I and II); with a change in particle diameter from 7 to 10 μm, the efficiency decreases by about 18%.

The retention behaviour of the peptides was studied using aqueous phosphate and acetate buffers as mobile phases. The elution orders were similar in the two mobile phases (see Table II), but the phosphate buffer permitted a better resolution of the peptides and a longer lifetime of the column; therefore, the dependences of the peptide retention on the experimental conditions [buffer concentration (Fig. 1), pH (Fig. 2) and the content of the methanol organic modifier (Fig. 3)] were studied with the phosphate mobile phase.

In ion-exchange separations, the retention is primarily determined by electrostatic interactions between the solute and the stationary phase; hence, the effect of the buffer concentration, *i.e.*, the mobile phase ionic strength, should predominate. All the dipeptides studied in this work contain glycine whereas the other amino acids differ in their side-chains. It can be seen from Fig. 1 that the retention of all the dipeptides decrease with increasing ionic strength, in agreement with the theory of ion exchange, except for the two substances containing free carboxylic groups, *i.e.*, Gly–Asp and Gly–Glu, where the dependence is reversed. These two substances are negatively charged under the given experimental conditions and thus cannot be retained

TABLE II

RETENTION DATA FOR DIPEPTIDES

Column I (see Table I); flow-rate, 0.5 ml/min.

Dipeptide	*Side-chain*	*Capacity ratio*	
		5mM phosphate	*2 mM acetate*
Gly–Asp	$-CH_2COO^-$	1.23	0.72
Gly–Glu	$-CH_2CH_2COO^-$	1.51	0.88
Gly–Gly	–H	1.93	1.83
Gly–Ala	$-CH_3$	1.98	–
Gly – Val	$-CH(CH_3)_2$	2.33	2.20
Gly–Met	$-CH_2CH_2SCH_3$	2.35	–
Gly–Leu	$-CH_2CH(CH_3)_2$	2.91	2.71
Gly–Phe	$-CH_2C_6H_5$	4.33	3.93
Gly–Tyr	$-CH_2C_6H_4OH$	5.32	4.87
Gly–Gly–Gly	–H	2.03	–
Gly–Gly–Gly–Gly	–H	2.05	–

through ion exchange on a cation exchanger; it seems that their retention is governed by hydrophobic interactions, possibly combined with a size-exclusion effect.

These dependences demonstrate the dual character of interactions on this stationary phase, ion exchange and hydrophobicity, as pointed out above. The peptides are separated into groups by ion exchange, whereas the separation within a group depends on the hydrophobicity of the amino acid side-chain. For example, the elution

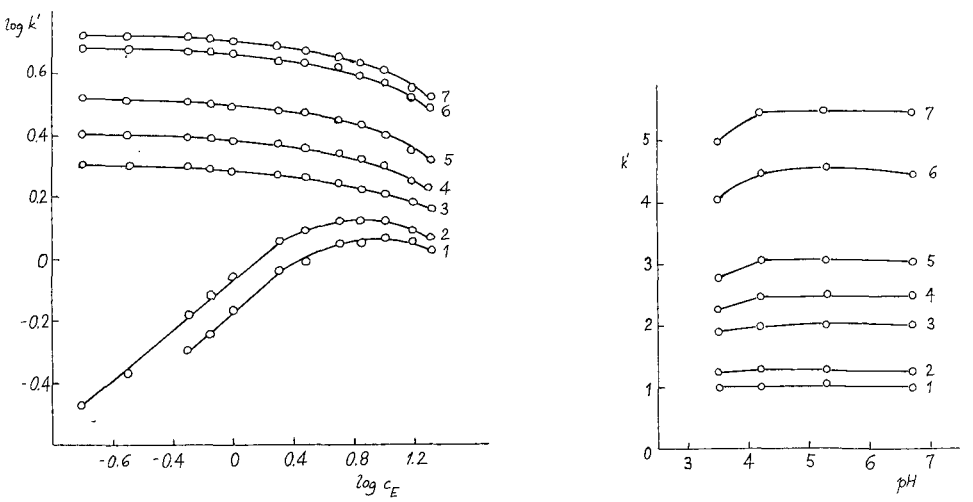

Fig. 1. Dependence of log k' on buffer concentration. Column IV; sodium phosphate buffer; flow-rate, 0.5 ml/min; pH 5.3; UV photometric detection at 210 nm. 1 Gly–Asp; 2 = Gly–Glu; 3 = Gly–Gly; 4 = Gly–Val; 5 = Gly–Leu; 6 = Gly–Phe; 7 = Gly–Tyr.

Fig. 2. Dependence of k' on mobile phase pH. Column I; 10^{-3} M sodium phosphate buffer; flow-rate, 0.5 ml/min; without methanol; UV photometric detection at 210 nm. Substances as in Fig. 1.

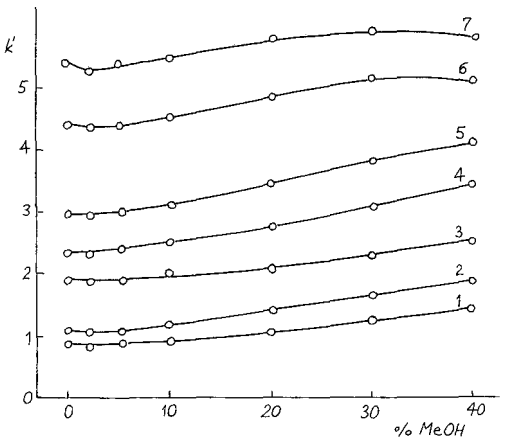

Fig. 3. Dependence of k' on the methanol (MeOH) content in the mobile phase (%, v/v). Conditions as in Fig. 2; pH, 5.3.

order follows the increasing side-chain hydrophobicity in the series Gly–Gly, Gly–Ala, Gly–Val, Gly–Leu and Gly–Phe, with the respective side-chains, –H, $-CH_3$, $-CH(CH_3)_2$, $-CH_2CH(CH_3)_2$ and $-CH_2C_6H_5$.

Vláčil and co-workers[26,27] proposed the following relationship for ion exchange on HEMA columns:

$$\log k' = p + q \log c_E$$

where k' is the capacity ratio, c_E is the concentration and p, and q are empirical constants. As can be seen from Fig. 1, this relationship only holds for low buffer concentrations, whereas a more complex mechanism operates at high concentrations. The side-chain character exerts a decisive effect on the separation, whereas the length of the peptide main chain is unimportant (see Table II, demonstrating poor resolution of Gly–Gly, Gly–Gly–Gly and Gly–Gly–Gly–Gly).

The dependences on pH (Fig. 2) and on methanol content in the mobile phase (Fig. 3) are not pronounced. The retention of Gly–Asp and Gly–Glu does not depend on pH within the region studied, whereas the retention of the other peptides increases slightly with increasing pH. The retention also increases slightly with increasing methanol content from 0 to 40% (v/v).

The plots of the log k' values against carbon number (n) in the peptide side-chain (Fig. 4) are linear for $n > 3$, in agreement with Molnár and Horváth[28].

The separation of dipeptides on the columns tested is shown in Fig. 5.

Detection

UV photometric detection at 210 nm, which is commonly used in the HPLC of peptides[1,3], was also employed. The limits of detection, *ca.* 1 ng in the volume injected, are in agreement with the values given in the literature. The detection sensitivity is highest for peptides with aromatic rings, such as Gly–Tyr and Gly–Phe.

An alternative detection technique is amperometry with a copper working elec-

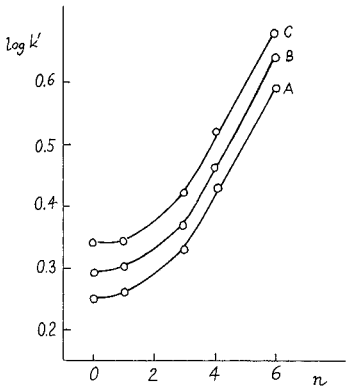

Fig. 4 Dependence of log k' on the number of carbon atoms in the dipeptide side-chain. Column I; flow-rate, 0.5 ml/min; UV photometric detection at 210 nm. Sodium phosphate buffer concentration: (A) $1 \cdot 10^{-2}$, (B) $5 \cdot 10^{-3}$ and (C) $2 \cdot 10^{-3}$ M.

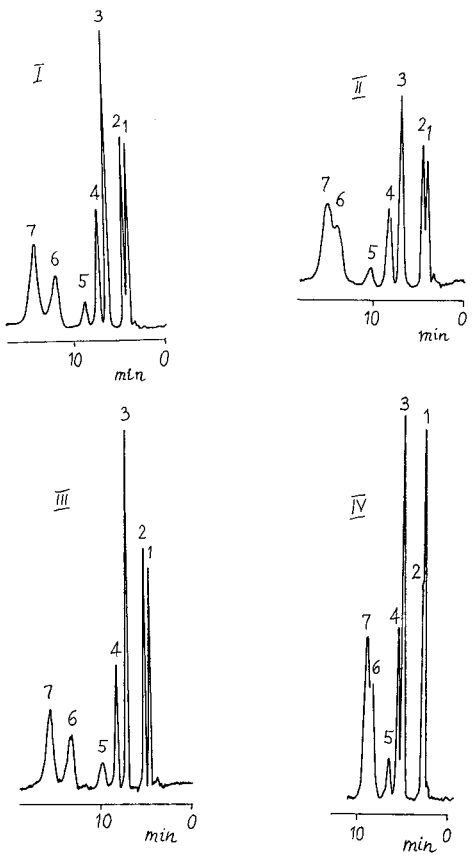

Fig. 5. Separation of dipeptides on the columns tested. 10^{-3} M sodium phosphate buffer (pH 5.3); flow-rate, 0.5 ml/min (columns I, II and IV) and 1.5 ml/min (column III). Substances as in Fig. 1.

trode[17–19]. As can be seen from Table III, the detection parameters for dipeptides are satisfactory, but the limits of detection are about one order of magnitude higher than those obtained by UV photometry. The sensitivity of detection decreases rapidly with increasing length of the peptide chain; among the tripeptides, only Gly–Gly–Gly and Leu–Gly–Gly can be detected, with a limit of detection of about 60 ng; the sensitivity is even poorer for tetrapeptides. The sensitivity of detection can be improved by using low mobile phase flow-rates, as the copper electrode current increases with decreasing flow-rate[18,19]; hence the use of micropacked and possibly also capillary columns would be advantageous. The main advantage of amperometric detection with a copper electrode is its high selectivity, hence the sample pretreatment is substantially simplified in many practical cases (see, *e.g.*, refs. 24 and 29).

TABLE III

CALIBRATION GRAPH PARAMETERS AND DETECTION LIMITS FOR VARIOUS PEPTIDES

(A) Amperometric detection with a copper electrode: mobile phase, aqueous 0.025 *M* NaH_2PO_4 (pH 6.8); flow-rate, 0.5 ml/min; sample size, 0.5 μl; electrode potential, +0.15 V; column I. (B) Tensammetric detection with a dropping mercury electrode: mobile phase, aqueous 0.1 *M* $NaClO_4$ (pH 5.0); flow-rate, 0.4 ml/min; sample size, 20 μl; electrode d.c. potential, −0.38 V; a.c. voltage amplitude, 30 mV; drop time, 3 s; without column.

Peptide	*A*			*B*		
	Slope [nA/(μg/μl)]	*Correlation coefficient*	*Detection limit (ng/μl)*	*Slope [nA/(μg/μl)]*	*Correlation coefficient*	*Detection limit (ng/μl)*
Glycine	1640	1.000	0.09	68.3	0.996	12
Gly–Gly	36.0	0.993	25	65.7	0.999	12
Gly–Gly–Gly	27.3	0.996	25	81.2	1.000	10
Gly–Gly–Gly–Gly	9.4	0.984	100	82.2	0.996	10
Gly–Asp	65.4	1.000	10	–	–	–
Gly–Ala	98.3	0.998	10	–	–	–
Gly–Val	51.7	0.999	10	–	–	–
Gly–Leu	31.7	0.999	25	–	–	–
Gly–Glu	84.8	0.999	10	–	–	–
Gly–Phe	48.8	0.998	25	–	–	–
Gly–Tyr	22.7	0.993	40	–	–	–
Gly–Met	89.9	0.994	10	–	–	–
Leu–Gly–Gly	18.7	0.996	50	487.0	1.000	1.7
Tyr–Ala–Gly–Phe–Cys(Me)	–	–	–	165.0	0.995	5.5
Insulin	–	–	–	13.4	0.994	59.5

Tensammetric detection at a mercury electrode, another selective detection method, was further investigated. It has been found that a hanging mercury drop cannot be used, as it rapidly becomes passivated and the measuring sensitivity and reproducibility are poor; the results are satisfactory with a dropping mercury electrode. The dependence of the response on the applied d.c. potential (Fig. 6) indicates that the optimum potential is in the range −0.36 to −0.40 V. The tensammetric response increases with increasing surface area of the mercury drop, with increasing drop time (up to a value of 3 s and then remains constant) and with increasing

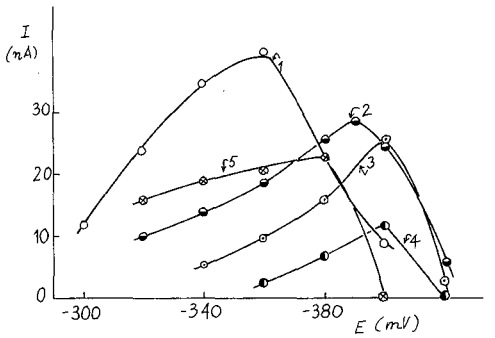

Fig. 6. Dependence of the tensammetric signal on the applied potential. Mobile phase, 0.1 *M* $NaClO_4$; flow-rate, 0.3 ml/min; pulse amplitude, 30 mV; drop time, 3 s. 1 = Gly–Gly; 2 = Leu–Gly–Gly; 3 = Gly–Gly–Gly; 4 = insulin; 5 = Glu–His–$ProNH_2$.

amplitude of the applied a.c. voltage. The maximum response was attained at a d.c. potential of −0.38 V, a drop time of 3 s and an a.c. voltage amplitude of 30 mV_r.

The dependence of the tensammetric response on the mobile phase pH is shown in Fig. 7. The optimum pH is 5–6, where the solutes are mostly uncharged, which supports their adsorption at the electrode surface. The tensammetric signal decreases with increasing flow-rate (Fig. 8), similarly to the amperometric signal at a copper electrode; the reason is the same in both instances, *i.e.*, a slow interaction (complexation reaction or adsorption) between the solutes and the electrode.

The tensammetric detection parameters are given in Table III. It can be seen that the detection sensitivity is comparable to that with amperometry at a copper electrode. On the other hand, tensammetric detection does not depend on the length of the peptide chain (*e.g.*, in the series Gly–Gly, Gly–Gly–Gly, and Gly–Gly–Gly–Gly). As can be expected, the sensitivity of tensammetric detection depends strongly

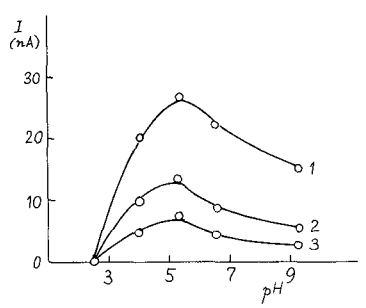

Fig. 7. Dependence of the tensammetric signal on the mobile phase pH. Applied potential, −380 mV; other conditions as in Fig. 6. 1 = Gly–Gly; 2 = glycine; 3 = insulin.

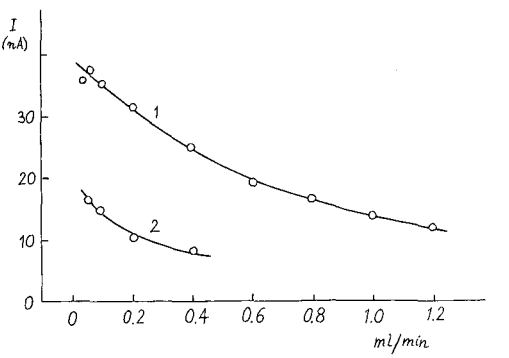

Fig. 8. Dependence of the tensammetric signal on the mobile phase flow-rate. Applied potential, −380 mV; flow-rate, 0.3 ml/min; other conditions as in Fig. 6. 1 = Gly–Gly; 2 = Gly–Gly–Gly–Gly.

on the mobile phase composition; mobile phases that do not contain surface-active components are generally preferable. In this work, measurement in the 0.1 *M* sodium perchlorate mobile phase yielded a signal twice as high as those in 0.025 *M* phosphate and acetate.

The reproducibilities of all the three detection techniques, *i.e.*, UV photometry, amperometry with a copper electrode and tensammetry, are similar, provided that each method operates under its particular optimum conditions. The relative standard deviations of the peak height were 1–2% at medium solute concentrations and increased to 5% close to the limit of detection.

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Note

Charge-transfer chromatographic study on inclusion complex formation between two hydroxypropyl-β-cyclodextrins and some chlorophenols

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Cyclodextrins (CDs) can form complexes with a wide variety of organic compounds[1,2], modifying their physico-chemical parameters, and are being increasingly applied in all types of chromatography[3–5]. In addition to solving separation problems, chromatographic techniques have been applied to determine the strength of various inclusion complexes[6]. Charge-transfer chromatography carried out on reversed-phase thin-layer chromatographic (TLC) plates has been used to study the inclusion complex formation of polymyxin[7], symmetric triazine[8], triphenylmethane[9], nitrostyrene[10] and barbituric acid derivatives[11]. Determination is based on the lipophilicity difference between the complexed and free forms of the guest compound[12]. A similar method was applied to determine the α-cyclodextrin complexes of 4-chloro- and 2,4,6-trichlorophenol[13].

The objectives of this investigation were to study the interaction of some chlorophenols with two hydroxypropyl-β-cyclodextrin derivatives and to elucidate the role of various chloro substitutions in the complex formation.

EXPERIMENTAL

The following chlorophenol derivatives were studied: 2-chloro-(I), 3-chloro-(II), 4-chloro-(III), 4-chloro-3-methyl-(IV), 2,4-dichloro-(V), 2,5-dichloro-(VI), 3,5-dichloro-(VII), 2,4,5-trichloro-(VIII) and 2,4,6-trichlorophenol (IX). Silufol UV254 TLC plates (Kavalier, Sklárny, Czechoslovakia) were impregnated with paraffin oil as described previously[7]. The chlorophenol derivatives were dissolved in acetone at a concentration of 2 mg/ml and 5 μl of each solution were spotted on the plates. As the aim was to study the complex formation between the chlorophenols and two hydroxypropyl-β-cyclodextrin (HPBCD) derivatives (average degree of substitution

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2.7 and 4.6) (henceforth called HPBCD 2,7 and HPBCD 4,6) and not to study the effect of HPBCDs on the separation of chlorophenols, the chlorophenols were spotted separately on the plates in each instance; in this manner the HPBCD: chlorophenol ratio was always identical for each chlorophenol derivative. This experimental design excluded the competition between the various chlorophenols for the cavities of HPBCD and their possible interaction with each other, which may influence the complex formation.

Methanol was chosen as the organic solvent miscible with water because it forms only weak inclusion complexes with β-cyclodextrins[14,15]. Methanol was incorporated in the eluent in the concentration range 0–30 vol.-% in steps of 5%. After development the plates were dried at 105°C and the chlorophenol spots and the HPBCD fronts were detected under UV light and with anthrone reagent, respectively. For each experiment five replicate determinations were carried out.

To separate the effect of methanol and HPBCD concentrations on the lipophilicity of chlorophenols, the following equation was fitted to the experimental data:

$$R_M = R_{M0} + b_1C_1 + b_2C_2 \quad (1)$$

where

R_M = actual R_M value of a compound determined at given methanol and HPBCD concentrations;

R_{M0} = R_M value of a compound extrapolated to zero methanol and HPBCD concentrations;

b_1 = decrease in the R_M value caused by a 1% increase in the methanol concentration in the eluent;

b_2 = decrease in the R_M value caused by 1 mM change in the concentration of HPBCD in the eluent;

C_1, C_2 = methanol and HPBCD concentration, respectively.

Eqn. 1 was applied separately for each compound and for both HPBCD derivatives.

To elucidate the role of lipophilicity in the inclusion complex formation, linear correlations were calculated between the R_{MO} values and the b_2 values for each RPTLC system:

$$b_2 = a + bR_{MO} \quad (2)$$

To compare the complex-forming capacity of various cyclodextrin derivatives, the complex stability values of HPBCDs were linearly correlated with the complex stability values of a water-soluble β cyclodextrin polymer[16].

RESULTS AND DISCUSSION

The mean $R_F \times 100$ values of chlorophenols are given in Table I. The R_F values increase in each instance with increase in methanol concentration, *i.e.*, these compounds do not show any anomalous retention behaviour in this concentration range that would invalidate the evaluation using eqn. 1. An increase in HPBCD concentra-

tion also caused an increase in R_F values, proving the complex (probably inclusion complex) formation. Interaction of the more hydrophilic HPBCDs with the chlorophenols reduces the lipophilicity of the latter.

The simultaneous effects of methanol and HPBCD 2,7 concentrations on the R_M values of 2,4-dichloro- and 2,4,6-trichlorophenols are shown in Figs. 1 and 2. In both instances the lipophilicity decreases with increasing methanol and HPBCD concentration; the effect depends on the type of compound and on the composition of the eluent.

The presence of HPBCDs in the eluent did not affect the compactness and symmetry of the peaks, as shown in Figs. 3–5. This observation is in good agreement with previous work[17], where β-cyclodextrin in the eluent did not influence the peak shape and symmetry markedly.

The parameters of eqn. 1 are compiled in Table II. The equation fits the experi-

TABLE I

R_F × 100 VALUES OF CHLOROPHENOLS

Eluent composition			*Compound*								
Methanol (%, v/v)	*Compound*	*Cyclodextrin concentration (mg/ml)*	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*
30	HPBCD 4,6	0	0.29	0.36	0.35	0.63	0.77	0.73	0.75	1.25	1.36
35		0	0.20	0.30	0.26	0.53	0.64	0.64	0.65	1.08	1.15
40		0	0.12	0.25	0.21	0.43	0.53	0.56	0.59	1.00	1.01
45		0	0.03	0.09	0.05	0.28	0.34	0.36	0.42	0.74	0.76
25		10	0.33	0.39	0.36	0.63	0.76	0.69	0.72	0.99	1.35
30		10	0.30	0.33	0.30	0.55	0.66	0.61	0.64	0.92	1.17
20		15	0.30	0.39	0.36	0.63	0.70	0.62	0.72	0.87	1.35
25		15	0.28	0.36	0.34	0.61	0.72	0.55	0.68	0.86	1.33
30		15	0.22	0.29	0.26	0.48	0.61	0.57	0.58	0.76	1.13
20		20	0.23	0.34	0.33	0.60	0.68	0.60	0.66	0.78	1.24
25		20	0.22	0.30	0.29	0.53	0.66	0.58	0.60	0.71	1.13
30		20	0.21	0.23	0.22	0.45	0.56	0.48	0.52	0.65	1.01
15		25	0.39	0.37	0.34	0.63	0.69	0.60	0.61	0.72	1.23
20		25	0.28	0.30	0.28	0.52	0.59	0.56	0.58	0.89	1.14
25		25	0.19	0.27	0.25	0.49	0.59	0.50	0.55	0.59	1.06
30		25	0.11	0.24	0.22	0.44	0.51	0.47	0.50	0.56	1.00
25	HPBCD 2,7	10	0.36	0.43	0.42	0.70	0.84	0.79	0.80	1.05	1.50
30		10	0.25	0.35	0.36	0.58	0.69	0.67	0.70	1.00	1.24
20		15	0.40	0.45	0.44	0.76	0.87	0.79	0.80	0.97	1.51
25		15	0.24	0.36	0.35	0.55	0.74	0.69	0.68	0.85	1.28
30		15	0.20	0.29	0.26	0.51	0.65	0.63	0.62	0.80	1.24
20		20	0.21	0.38	0.38	0.69	0.72	0.69	0.71	0.70	1.44
25		20	0.15	0.29	0.27	0.50	0.67	0.59	0.59	0.67	1.13
30		20	0.14	0.24	0.22	0.45	0.57	0.51	0.54	0.74	1.09
15		25	0.27	0.37	0.38	0.58	0.75	0.68	0.68	0.71	1.35
20		25	0.19	0.34	0.34	0.59	0.71	0.62	0.66	0.71	1.32
25		25	0.08	0.23	0.23	0.47	0.56	0.54	0.54	0.61	1.08
30		25	0.00	0.14	0.12	0.32	0.48	0.42	0.43	0.47	0.92

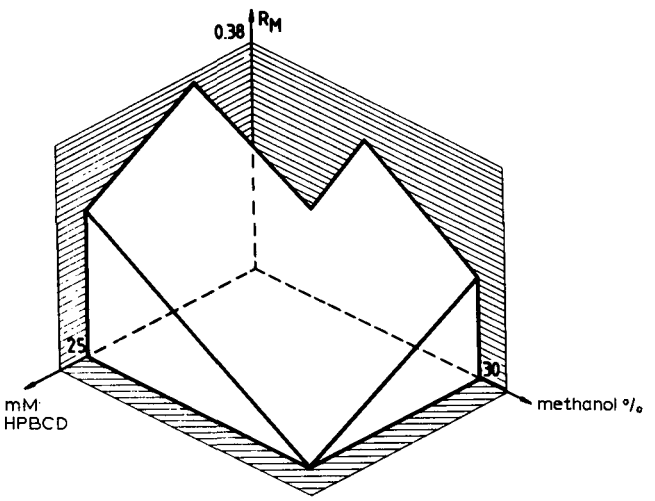

Fig. 1. Effect of methanol and HPBCD 4,6 concentrations on the R_M value of 2,4-dichlorophenol.

mental data well, the significance levels in each instance being over 99.9%; the ratios of variance explained were about 75–90% (see r^2 values). The complex stability (b^2 values) increases with increasing number of substituents. The monohalogenated derivatives form the weakest and the trichlorinated derivatives the strongest complexes. Methyl substitution has a similar influence to chloro substitution on the complex

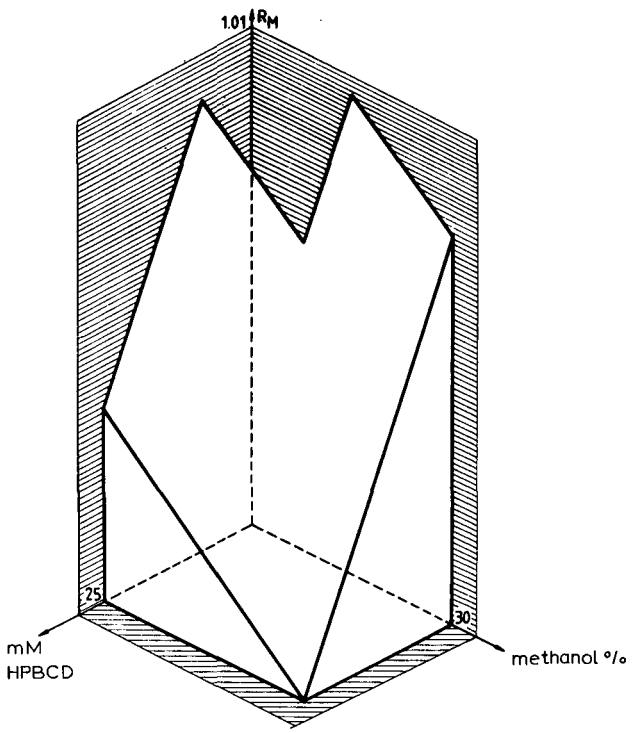

Fig. 2. Effect of methanol and HPBCD 4,6 concentrations on the R_M value of 2,4,6-trichlorophenol.

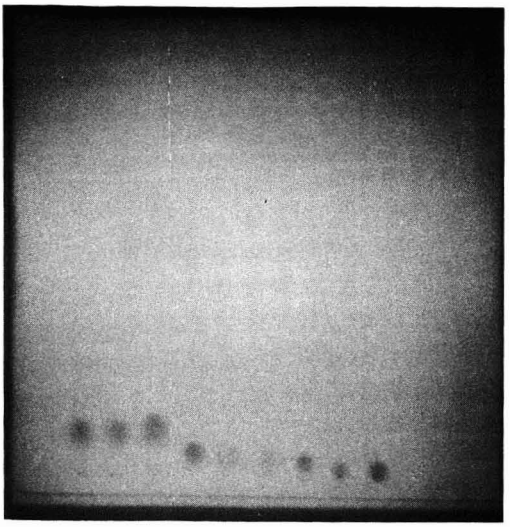

Fig. 3. UV detection of chlorophenols with water–methanol (4:1) as eluent. Chlorophenols I–IX appear consecutively from left to right.

stability. The normalized slopes (b') show that a change in HPBCD concentration has a similar effect to a change in methanol concentration on the retention of chlorophenols.

The R_F values of the front of the eluent additives are given in Table III. Each additive front is well ahead of the chlorophenol spots, *i.e.*, the differences observed between the retention behaviours of chlorophenols in various eluent systems are really caused by the presence of HPBCD.

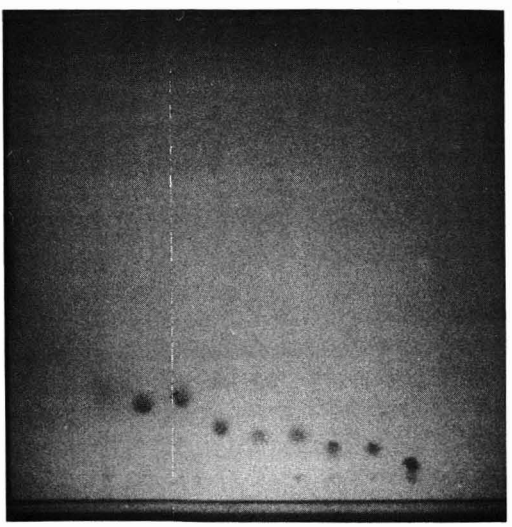

Fig. 4. UV detection of chlorophenols with water–methanol (4:1) + 25 m*M* HPBCD 2,7 as eluent. Chlorophenols as in Fig. 3.

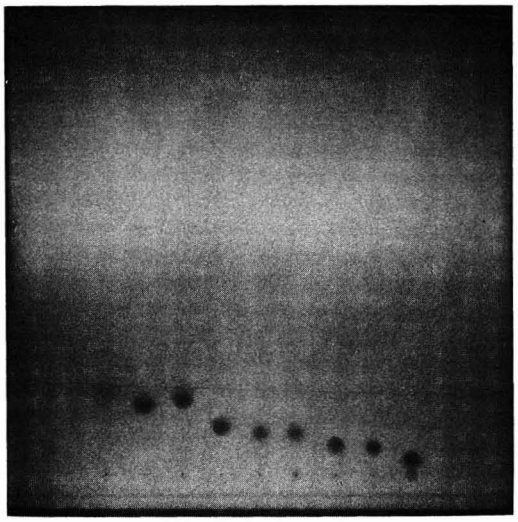

Fig. 5. UV detection of chlorophenols with water–methanol (4:1) + 25 m*M* HPBCD 4,6 as eluent. Chlorophenols as in Fig. 3.

TABLE II

CORRELATIONS BETWEEN R_M VALUES OF CHLOROPHENOLS AND CONCENTRATIONS OF METHANOL AND HPBCD IN THE ELUENT

Eqn. 1: $n = 16$; $F_{99,9\%} = 12.31$; $t_{99.9\%} = 4.22$.

Parameter	*Cyclodextrin*	*Compound*								
		I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*
R_{M0}	HPBCD 4,6	0.74	0.76	0.73	1.13	1.27	1.12	1.18	1.73	2.16
$-b_1 \cdot 10^3$		15.2	13.2	13.4	17.4	18.2	14.4	15.1	19.3	28.4
$-b_2 \cdot 10^3$		6.23	6.32	5.89	8.41	9.73	10.4	10.7	25.4	15.6
$r^2(\%)$		82.21	84.80	84.30	90.13	77.75	75.13	83.96	83.81	83.99
$s \cdot 10^2$		4.11	3.19	3.38	3.29	5.46	4.76	3.78	7.98	6.97
F		30.04	36.26	34.91	59.36	22.72	19.63	34.02	35.66	34.09
t_1		7.37	8.34	8.04	10.68	6.71	6.08	8.06	4.89	8.23
t_2		3.83	5.01	4.41	6.46	4.50	5.51	7.14	8.05	5.66
b_1'		1.28	1.34	1.31	1.38	1.30	1.25	1.33	0.81	1.36
b_2'		0.67	0.81	0.72	0.84	0.88	1.13	1.18	1.33	0.93
R_{M0}	HPBCD 2,7	0.90	0.93	0.97	1.36	1.52	1.41	1.39	1.81	2.50
$-b_1 \cdot 10^3$		19.1	17.7	19.4	23.3	24.6	21.8	20.5	20.6	37.2
$-b_2 \cdot 10^3$		12.6	9.72	9.73	12.8	12.5	13.4	13.4	28.6	18.2
$r^2(\%)$		84.59	89.80	90.79	87.68	89.65	88.96	90.06	87.84	88.98
$s \cdot 10^2$		4.60	3.34	3.51	4.91	4.73	4.29	3.84	7.68	7.46
F		35.68	57.24	64.09	46.25	56.32	52.38	58.89	46.95	52.50
t_1		8.38	10.67	11.17	9.59	10.49	10.22	10.78	5.39	10.07
t_2		6.91	7.35	7.01	6.59	6.69	7.90	8.82	9.41	6.18
b_1'		1.36	1.40	1.40	1.39	1.39	1.40	1.40	0.78	1.38
b_2'		1.12	0.97	0.88	0.95	0.89	1.08	1.15	1.35	0.85

TABLE III

R_F VALUES OF HPBCD 2,7 AND HPBCD 4,6 FRONTS IN VARIOUS ELUENT SYSTEMS

Eluent composition		*R_F value*	
Methanol (%, v/v)	*HPBCD 2,7 or HPBCD 4,6 (mM)*	*HPBCD 2,7*	*HPBCD 4,6*
25	10	0.74	0.42
30	10	0.82	0.46
20	15	0.80	0.47
25	15	0.81	0.46
30	15	0.85	0.52
20	20	0.84	0.52
25	20	0.89	0.53
30	20	0.89	0.56
15	25	0.87	0.52
20	25	0.89	0.60
25	25	0.88	0.56
30	25	0.92	0.56

The correlation coefficients of eqn. 2 were 0.7171 and 0.6365 for HPBCD 4,6 and 2,7, respectively, which suggests that the lipophilicity of chlorophenol derivatives does not explain adequately the strength of interaction and the steric parameters are probably more important in the inclusion complex formation.

Significant linear correlations were found between the complex stability values of various cyclodextrin derivatives:

$$b_{\text{HPBCD 2,7}} = -0.88 + 0.66 b_{\text{SCDP}};\ r = 0.8803$$

$$b_{\text{HPBCD 4,6}} = -6.22 + 0.73 b_{\text{SCDP}};\ r = 0.9197$$

where SCDP is a water-soluble β-cyclodextrin polymer.

The results demonstrate that the various substituents on the β-cyclodextrin ring modify the complex-forming capacity, but the order of complex stabilities remains the same, that is, the interaction in each instance is governed by the insertion of guest molecules in the cyclodextrin cavity.

CONCLUSIONS

Chlorophenol derivatives form inclusion complexes with hydroxypropyl-β-cyclodextrins and the complex stability increases with increase in the number of chloro substituents whereas the position of substitution has smaller effect on the complex stability. Charge-transfer chromatography proved to be a suitable method for studying such interactions.

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Simultaneous determination of insecticides, acaricides and fungicides by thin-layer chromatography

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ABSTRACT

A method is described for the determination of residual amounts of some insecticides, acaricides and fungicides in fresh and processed fruits. Optimum conditions were established for the extraction of eighteen pesticides, purification of the extracts by column chromatography and subsequent two-dimensional thin-layer chromatography on silica gel GF_{254} with cyclohexane–acetone (10:1) and light petroleum–benzene–ethanol (65:30:5) as solvents and with detection under UV light at 254 and 366 nm followed by a 0.1% solution of bromophenol blue.

INTRODUCTION

The wide application of pesticides requires methods for the detection and determination of pesticide residues in agricultural products and foodstuffs. Thin-layer chromatography (TLC) has proved advantageous for the determination of modern perticides belonging to different chemical classes[1–7].

The aim of this work was to develop a analytical procedure based on TLC for rapid determining various insecticides, acaricides and fungicides in fresh and processed fruits.

EXPERIMENTAL

Reagents

Standard acetone solutions (1000 $\mu g/cm^3$) of the following pesticides were prepared: dimethoate ($C_5H_{12}NO_3PS_2$), tetrachlorvinphos ($C_{10}H_9Cl_4O_4P$), pyrazophos ($C_{14}H_{20}N_3O_5PS$), diazinon ($C_{12}H_{21}N_2O_3PS$), phozalone ($C_{12}H_{15}ClNO_4PS_2$), pyrimiphosmethyl ($C_{11}H_{20}N_3O_3PS$), fenitrothion ($C_9H_{12}NO_5PS$), chlorpyriphos ($C_9H_{11}Cl_3NO_3PS$), deltametrin ($C_{22}H_{19}Br_2NO_3$), silhaletrin ($C_{23}H_{19}F_3ClNO_3$), triflumisol ($C_{15}H_{15}F_3ClN_3O$), fenarimol ($C_{17}H_{12}Cl_2N_2O$), iprodion ($C_{13}H_{13}Cl_2N_3O_3$), vinclosolin ($C_{12}H_9Cl_2NO_3$), hexathiazox ($C_{17}H_{21}ClN_2O_2S$),

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TABLE I

R_F VALUES OF INSECTICIDES, FUNGICIDES AND ACARICIDES WITH DIFFERENT SOLVENT SYSTEMS ON SILICA GEL G

Pesticide	R_F *value*				
	(1) Cyclohexane–acetone (10:1)	*(2) Light petroleum–benzene–ethanol (65:30:5)*	*(3) Benzene–acetone (95:5)*	*(4) Hexane–tetrachloromethane–ethyl acetate (65:30:5)*	*(5) Cyclohexane–benzene–acetone (5:2:1)*
Insecticides					
Dimethoate	0.05	0.07	0.03	0.03	0.07
Tetrachlorvinphos	0.19	0.22	0.12	0.05	0.27
Pyrazophos	0.29	0.27	0.43	0.07	0.44
Diazinon	0.45	0.29	0.53	0.13	0.53
Phozalone	0.31	0.33	0.55	0.17	0.50
Pyrimiphos-methyl	0.52	0.41	0.79	0.25	0.62
Fenitrothion	0.33	0.43	0.87	0.20	0.53
Chlorpyriphos	0.73	0.69	0.90	0.52	0.75
Deltametrin	0.39	0.56	0.78	0.45	0.70
Sihaletrin	0.30	0.59	0.83	0.48	0.66
Fungicides					
Triflumisol	0.12	0.19	0.14	0.04	0.20
Fenarimol	0.15	0.17	0.16	0.07	0.25
Iprodion	0.17	0.24	0.40	0.13	0.35
Vinclosolin	0.45	0.44	0.85	0.56	0.66
Guazatin	0.53	0.39	0.82	0.50	0.78
Acaricides					
Hexathiazox	0.44	0.31	0.76	0.29	0.70
Chlofentisin	0.39	0.42	0.86	0.47	0.70
Flubenzimin	0.36	0.51	0.90	0.60	0.74

chlofentisin ($C_{12}H_{11}Cl_2N_4$), flubenzin ($C_{17}H_{10}F_6N$) and guazatin ($C_{15}H_{33}N_4O_6$). The TLC supports were silica gel G and silica gel GF_{254} (Merck). The solvent systems used are given in Table I. Detection was effected with (1) 0.1% bromophenol blue (BPB) solution prepared by dissolving 0.1 g of BPB and in 100 cm^3 of 1% $AgNO_3$ solution in 0.5 *M* ammonia, (2) UV light at 254 and 366 nm, (3) Dragendorff reagent[8] and (4) iodine vapour.

Procedure

A solutions containing 25 μg of a pesticide was mixed with 50 g of apple homogenate free from pesticides or with apple juice. All pesticides were extracted with 130 cm^3 of methanol for 50 min.

Each extract was filtered through a silica gel layer and the filtrate was mixed with 150 cm^3 of 10% sodium chloride solution. The pesticides were extracted twice from the solution with 80 cm^3 of chloroform and the extracts were evaporated to 4–5 cm^3 and passed down a chromatographic column packed with 5 g of adsorbent consisting of sodium sulphate, Florisil, Celite and charcoal (1:1:0.5:0.1). The pesticides in the column were eluted using 90 cm^3 of chloroform–diethyl ether (9:1). The eluate was evaporated to dryness at 40°C and the dry residue was dissolved in 0.5 cm^3 of acetone. A 250-μl aliquot of the acetone solution was spread on a chromatographic plate precoated with silica gel GF_{254}. A standard mixture of the pesticides was spread on another plate.

The pesticides were separated using two-dimensial TLC with the solvent systems cyclohexane–acetone (10:1) and light petroleum–benzene–ethanol (65:30:5). To identify the separated spots, the plates were first irradiated with UV light at 254 nm, which developed all pesticides except vinclosolin and dimethoate; the vinclosolin spot was then revealed under UV light at 366 nm and that of dimethoate by spraying with 0.1% BPB solution. The areas of the spots developed on the test plates and the plate with the standard mixture were compared in order to determine the pesticide contents in the samples.

RESULTS AND DISCUSSION

TLC has a number of advantages for determining a wide range of pesticides with different structures, owing to the rapidity and efficiency of separation and identification.

To establish the optimum conditions for the TLC analysis of the pesticides, a number of tests were performed on common chromatographic supports such as silica gel G, alumina, cellulose, Florisil and polyamide[1–8]. Silica gel G proved to be the most suitable and was adopted in subsequent work. The mobile phase for separating the pesticides under consideration was chosen after testing about 50 combinations of solvents, some of which are used in pesticide analysis and others in chromatographic analyses of similar compounds[1–11]. The most suitable are given in Table I. The results indicate that the most suitable systems for insecticides are 1 and 2, for acaricides 2 and 4 and for fungicides 5 and 2.

As none of the solvent systems could be used for the simultaneous determination of the whole combination of eighteen pesticides, two-dimensional TLC was tested with systems 1 and 2 successively. The latter was suitable for all three groups of

TABLE II

SENSITIVITY (μg) TO IDENTIFICATION OF INSECTICIDES, FUNGICIDES AND ACARICIDES WITH DIFFERENT DETECTION METHODS

Two-dimensional TLC on silica gel G with cyclohexane–acetone (10:1) and light petroleum–benzolene–ethanol (65:30:5)

Pesticide	*Detection*							
	0.1% BPB		*UV light (254 nm)*[a]		*Dagendorff reagent*		*Iodine vapour*	
	Sensitivity	*Colour of spot on pale lilac background*	*Sensitivity*	*Colour of spot on yellow-green background*	*Sensitivity*	*Colour of spot on pale yellow background*	*Sensitivity*	*Colour of spot on white background*
Insecticides								
Dimethoate	2	Dark blue	–	–	–	–	10	Brown
Tetrachlorvinphos	1	White	2	White	2	Orange	5	Brown
Pyrazophos	0.5	Blue-green	0.5	Light blue	2	Red-brown	2	Brown
Diazinon	1	White	5	Violet	0.5	Red-brown	10	Brown
Phozalone	2	Dark blue	5	Blue	5	Orange	5	Brown
Pyrimiphos-methyl	2	Dark lilac	1	Light blue	0.5	Red-brown	5	Brown
Fenitrothion	1	Dark lilac	1	Violet	5	Orange	10	Brown
Chlorpyriphos	5	Dark lilac	2	Blue	10	Orange	10	Brown
Deltametrin	1	Dark blue	5	Light blue	5	Orange	10	Brown
Sihaletrin	1	Dark blue	5	Light blue	10	Orange	10	Brown
Fungicides								
Triflumisol	2	Dark blue	2	Lilac	2	Red-brown	5	Brown
Fenarimol	0.5	Blue-green	2	Lilac	1	Red-brown	5	Brown
Iprodion	2	White	5	Blue	10	Orange	10	Brown
Vinclosolin	0.5	Dark lilac	2	Light blue (366 nm)	5	White	10	Brown
Guazatin	2	White	5	Lilac	10	Orange	20	Brown
Acaricides								
Hexathiazox	1	White	5	White	10	Orange	20	Brown
Chlofentisin	5	Dark lilac	0.5	Dark red	2	Red-brown	5	Brown
Flubenzimin	2	Yellow-green	2	Dark lilac	5	White	10	Brown

[a] Chromatographic support silica gel GF_{254}.

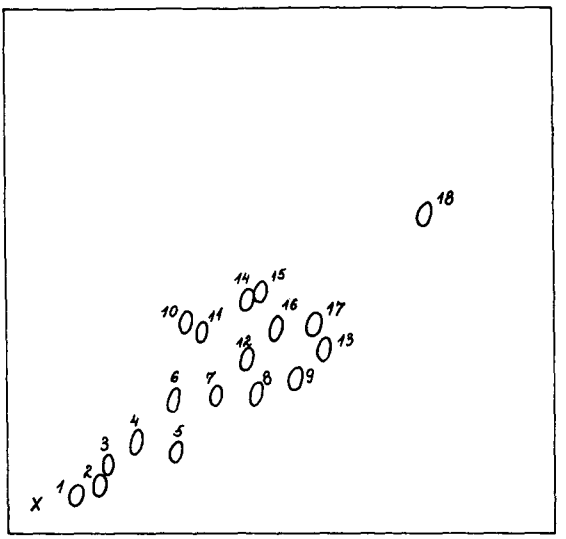

Fig. 1. Two-dimensional chromatogram of a mixture of 18 pesticides (10 μg each) on silica gel GF_{254}. Solvent systems: cyclohexane–acetone (10:1) and light petroleum–benzene–ethanol (65:30:5). Detection UV light (254 and 366 nm) and 0.1% BPB. 1 = Dimethoate; 2 = triflumisol; 3 = fenarimol; 4 = tetrachlorvinphos; 5 = iprodion; 6 = pyrazophos; 7 = phozalone; 8 = fenitrothion; 9 = flubenzimin; 10 = diazinon; 11 = hexathiazox; 12 = chlofentisin; 13 = sihaletrin; 14 = pyrimiphos methyl; 15 = guazatin; 16 = vinclosolin; 17 = deltametrin; 18 = chlorpyriphos.

TABLE III

ANALYTICAL RECOVERY AND DETECTION LIMIT IN TLC OF PESTICIDES IN APPLES

Pesticide	*Standard addition (mg kg^{-1})*	*Analytical recovery for apples (n = 12) (%)*	*Detection limit (mg kg^{-1})*
Diazinon	0.5	72	0.20
Dimethoate	0.5	80	0.08
Pyrimiphosmethyl	0.5	88	0.04
Chlorpyriphos	0.5	84	0.08
Tetrachlorvinphos	0.5	88	0.08
Phozalone	0.5	72	0.20
Fenitrothion	0.5	80	0.04
Pyrazophos	0.5	96	0.02
Deltametrin	0.5	80	0.20
Sihaletrin	0.5	72	0.20
Fenarimol	0.5	84	0.08
Vinclosolin	0.5	88	0.08
Triflumisol	0.5	80	0.08
Iprodion	0.5	72	0.20
Guazatin	0.5	80	0.20
Flubenzimin	0.5	80	0.08
Hexathiazox	0.5	76	0.20
Clofentisin	0.5	96	0.02

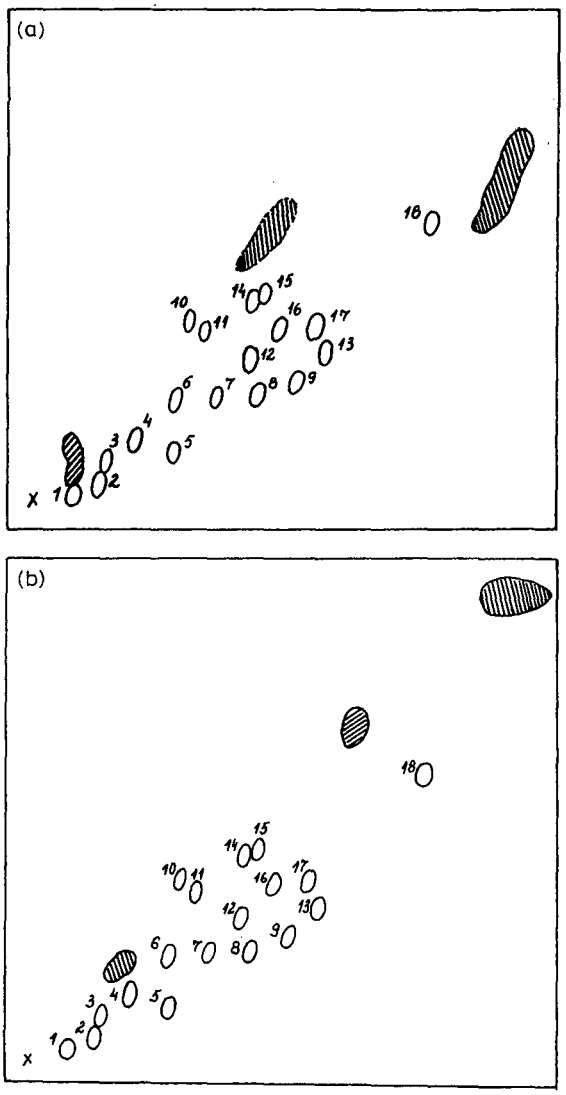

Fig. 2. Chromatograms of purified extract of (a) apples and (b) apple juice containing 18 pesticides (0.5 mg kg^{-1}) on silica gel GF_{254}.

pesticides, whereas system 1 ensured the best separation of the insecticides. Fig. 1 shows the results obtained from two-dimensional TLC on silica gel GF_{254}.

The detection reagents found to be suitable for the spots of the pesticides under consideration are listed in Table II. It can be seen that the highest sensitivity was obtained with identification using 0.1% BPB solution and UVirradiation at 254 nm. They produced intact spots of regular shape and their areas and intensities were proportional to the amounts of pesticides.

The results in Tables I and II indicate that the optimum conditions for the simultaneous TLC determination of the eighteen pesticides are the following: silica gel GF_{254} as the chromatographic support; two-dimensional chromatography for about 100 min with cyclohexane–acetone (10:1) and light petroleum–benzene–ethanol (65:30:5) and detection by successive UV irradiation at 254 and 366 nm for vinclosolin followed by spraying with 0.1% BPB solution for dimethoate.

Fig. 2 presents chromatograms of purified extracts of apples and apple juice (50 g) containing 0.5 ppm of each of the studies pesticides. Table III gives the results for pesticides determined by the standard additions method in apples and apple juice.

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Some possibilities of combining high-performance liquid chromatography with isotachophoresis for the trace determination of ionogenic compounds present in complex matrices

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ABSTRACT

The use of reversed-phase high-performance liquid chromatography (RP-HPLC) for sample preparation in capillary isotachophoresis (ITP) was investigated. Asulam (a pre- and post-emergent herbicide) served as a model analyte and soil extracts represented complex ionic matrices. Very efficient clean-up of the samples was achieved by using the ion-suppression mode of the chromatographic separation and hence, positive systematic errors in the ITP determination of asulam could be reduced to levels equivalent to 8–12 ppb (10^9) of the analyte depending on the extraction procedure employed. As the number of sample handling steps was small, high recoveries (95–105%) of the complete analytical procedure were typical. The proposed combination of the separation methods seems very promising for the trace determination of ionogenic compounds present in complex biological and environmental matrices.

INTRODUCTION

In the determination of trace ionogenic constituents present in complex ionic matrices by capillary isotachophoresis (ITP), suitable sample preparation procedures may become essential for achieving desired detection limits and/or for decreasing systematic analytical errors due to co-migrating matrix constituents. Liquid–liquid extraction (*e.g.*, refs. 1–3), solid-phase extraction (SPE) on suitable chromatographic

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sorbents (*e.g.*, refs. 4 and 5), coprecipitation[6,7] and the use of ion-exchange and chelating resins (*e.g.*, refs. 8–10) have mainly been proposed for sample clean-up and/or for trace enrichment before the final ITP analysis. Special attention was paid to the use of affinity sorbents in a combination with ITP[11]. Derivatization combined with these sample preparation procedures is also convenient, especially when selective detectors are employed for the evaluation of the analysis[12,13].

The above simple sample preparation procedures are less effective when the analyte and some of the matrix constituents have similar physico-chemical properties. For example, problems are encountered in practice when relatively hydrophilic ionogenic compounds present in aqueous sample solutions are of analytical interest. Preparative ITP[4,15], ITP on columns packed with granulated gels or with "inert" particular materials[16] and capillary zone electrophoresis[17,18] have been proposed for solving these problems in high-performance liquid chromatography (HPLC). On the other hand, gel chromatography was convenient for the fractionation of uraemic sera before analysis by ITP and by other high-performance separation methods[19,20]. Also, ITP determination of peptides in HPLC fractions is useful in the control of peptide synthesis (*e.g.*, ref. 21). In spite of a widespread use of HPLC, however, this technique was not considered for sample preparation in trace ITP analysis. This is surprising when the following facts are taken into account: (a) as the separation principles of ITP and HPLC are different it is reasonable to expect that in their sequential use working conditions giving non-correlated qualitative characteristics of the separands (retention time *vs.* effective mobility) can be found easily; (b) for example, by using various modes of reversed-phase (RP) HPLC, gel chromatography and ion-exchange chromatography on low-capacity sorbents, the mobile phases employed are often compatible with ITP electrolyte systems; (c) for many types of ionogenic compounds it is possible to use various alternatives of their liquid chromatographic separations so that an appropriate possibility for the sample preparation can be found easily; and (d) as a simple configuration of the chromatographic instrument can be sufficient for this purpose there are hardly any economic restrictions concerning the routine use of HPLC for sample preparation in ITP.

The aim of this work was to investigate some practical aspects of the use of RP-HPLC for sample preparation in ITP. The results presented were obtained with asulam [methyl (4-aminosulphonyl)carbamate] as a model analyte, and soil extracts served as typical complex ionic sample matrices. Asulam was chosen as in previous work[15] it served as a model analyte in illustrating the practical capabilities of preparative ITP as a sample preparation method for analytical ITP and HPLC. Hence the present results obtained with the sequence HPLC–ITP may in some respects be considered as complementary to the reverse sequence (ITP–HPLC) in order to compare their practical advantages and disadvantages.

EXPERIMENTAL

Instrumentation

A CS Isotachophoretic Analyzer (VVZ PJT, Spišská Nová Ves, Czechoslovakia) was assembled in the column-coupling configuration of the separation unit[22,23] using modules provided by the manufacturer. The analytical column was provided with an on-column UVD1 photometric detector (VVZ PJT) and detection was carried out at

254 nm. The samples were injected with the aid of a 30-μl sampling valve. An HP 3390A reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.) was used for signal evaluation.

An isocratic liquid chromatographic system consisting of an HPP 4001 pump (Laboratorní přístroje, Prague, Czechioslovakia), a Valco sampling valve provided with a 30-μl sample loop (Valco, Houston, TX, U.S.A.) and an LCD 2563 UV–VIS detector (Laboratorní přístroje) set at 254 nm was used. The signal from the detector was registered by a TZ 4200 line recorder (Laboratorní přístroje) and was evaluated by a Minigrator (Spectra-Physics, Santa Clara, CA, U.S.A.). Compact glass columns (150 × 3.3 mm I.D.) (Tessek, Prague, Czechoslovakia) were laboratory packed with 10-μm irregular Silasorb-phenyl sorbent (Lachema, Brno, Czechoslovakia). The columns were kept at 25 ± 0.5°C as described[24]. Water acidified with formic acid to pH 3.3 and mixed with methanol (75:25) served as the mobile phase in the chromatographic experiments at a flow-rate of 0.5 ml/min.

Chemicals

Chemicals used for the preparation of the leading and terminating electrolyte solutions were obtained from Serva (Heidelberg, F.R.G.), Sigma (St. Louis, MO, U.S.A.) and Lachema. Some were purified by conventional methods. Discrete spacers for ITP analyses as proposed by Madajová *et al.*[25] were employed. Hydroxyethylcellulose 4000 obtained from Sigma was purified on a mixed-bed ion exchanger (Amberlite MB-1; BDH, Poole, U.K.).

Water delivered by a Rodem-1 two-stage demineralization unit (OPP, Tišnov, Czechoslovakia) was further purified by circulation through laboratory-made polytetrafluorethylene (PTFE) cartridges packed with Amberlite MB-1. Only freshly recirculated water was employed for the preparation of the mobile phase, ITP electrolyte solutions and sample solutions. Doubly glass-distilled methanol of analytical-reagent grade (Lachema) was used throughout.

Asulam and sulphanilamide were kindly provided by the Residue Laboratory of the Research Institute of Chemical Technology (Bratislava, Czechoslovakia). Soil samples were obtained from the Centre of Soil Fertility (Bratislava, Czechoslovakia).

HEMA-cart DEAE and Silica-cart C_{18} disposable minicolumns were obtained from Tessek.

Soil extraction procedures and preparation of the extract for analysis

Methanolic extraction. A 50-g amount of air-dried soil was shaken with 100 ml of methanol for 5 h. The extract was filtered through a dense filter-paper and then through a 0.45-μm PTFE membrane filter (Schleicher and Schüll, Dassel, F.R.G.). An aliquot of the filtrate was concentrated to one third of the original volume under a stream of nitrogen and the residue was made up to this volume with water before the analysis. Soil samples fortified with asulam were treated in the same way.

A 50-ml aliquot of the filtered extract was evaporated to dryness under a stream of nitrogen to determine the amount of extracted material. For the soil samples used in this work and extracted with methanol it was *ca.* $2.5 \cdot 10^{-3}$% of the amount of the sample.

Extraction with aqueous borate buffer. A 50-g amount of air-dried soil was shaken with 100 ml of 10^{-2} *M* aqueous disodium tetraborate solution (pH 9.05) for 90

min. The extract was acidified with formic acid to pH $\approx$ 4.5 (part of the colloidal material coagulated) and filtered as above. In this way 0.9% of the sample taken for the analysis was extracted into the aqueous solution.

The extract obtained with borate buffer was further cleaned up by using the following procedure[26] to protect the HPLC columns from losses of their performance characteristics: (i) 17 ml of the filtered extract were percolated through a HEMA-cart DEAE minicolumn (the sorbent bed was washed successively with 5-ml volumes of 10^{-2} *M* aqueous disodium tetraborate solution and water immediately before use); (ii) the sorbent was washed with 2 ml of water to remove the unretained part of the sample; (iii) the analyte was eluted with 6 ml of $5 \cdot 10^{-2}$ *M* sulphuric acid; (iv) asulam from the desorbate was retained on a Silica-cart C_{18} column (the sorbent bed was washed successively with 5-ml volumes of methanol and water before sample application); (v) the sorbent bed was washed with 2 ml of $5 \cdot 10^{-2}$ *M* sulphuric acid to remove unretained matrix constituents; and (vi) asulam was backflushed with 1.2 ml of water–methanol (2:1, v/v).

RESULTS AND DISCUSSION

ITP and HPLC conditions

The composition of the operational (electrolyte) system used for the ITP experiments is given in Table I. The pH of the leading electrolyte was chosen in such a way that the number of isotachophoretically migrating matrix constituents was suppressed while the analyte having pK_a = 4.82 migrated spaced by *n*-propyl succinate and butyrate (see Fig. 1) to achieve favourable conditions for its quantitation by the spike method[27]. A higher content of methanol in the terminating electrolyte was necessary to avoid losses of the injected samples containing methanol due to different densities of the stacked solutions (vertical alignment of the ITP separation compartment).

Under these ITP working conditions we could detect and determine with confidence 200 pg of asulam (see Fig. 1). The detection limit for a signal-to-noise ratio

TABLE I

OPERATIONAL SYSTEM EMPLOYED IN THE ITP ANALYSES

The driving currents were 150 and 30 μA for the pre-separation and analytical columns, respectively.

Parameter	*Electrolyte*	
	Leading	*Terminating*
Solvent	Water–methanol	Water–methanol
Proportions	90:10	75:25
Anion	Cl^-	*n*-Caproate
Concentration (m*M*)	5	5
Counter ion	BALA[a]	BALA[a]
pH	3.85	$\approx$ 4
Additive	HEC[a]	–
Concentration (%, w/v)	0.1	–

[a] BALA = β-Alanine; HEC = hydroxyethylcellulose.

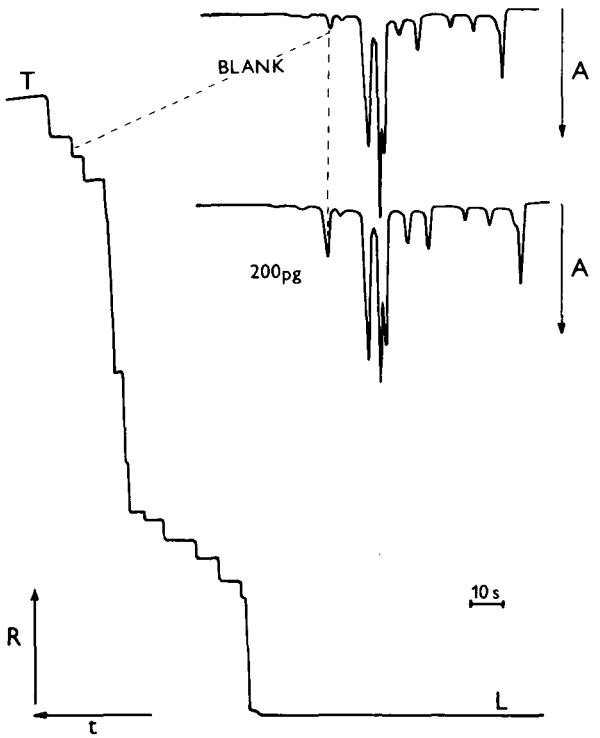

Fig. 1. Isotachopherograms from the analysis of 200 pg of asulam. In addition to the analyte, the injected sample solution contained 10^{-4} *M* sodium pyrophosphate (to eliminate losses of the analyte from adsorption on the surface of the sample handling devices) and a mixture of discrete spacing constituents (each at *ca.* 10^{-4} *M* concentration). In the blank run the same sample without the analyte was injected. The migration position of asulam was spaced by *n*-propyl succinate (a front spacing constituent in the direction of migration) and by butyrate (a rear spacer) as marked by dashed lines. The driving currents were 150 and 30 μA in the preparative and analytical columns, respectively. For the composition of the electrolyte system, see Table I. L and T = leading and terminating zones, respectively. *A*, *R* and *t* = increasing light absorption, resistance and time, respectively.

of 2 was *ca.* 75 pg (the noise was determined by run-to-run fluctuations of the peak area corresponding to the UV-absorbing impurities originating from the electrolyte solutions and migrating between the same spacing constituents as the analyte). This decrease in the detection limit in comparison with the previously estimated value[15] can be ascribed to the use of discrete spacers instead of a "continuous" spacing mixture. In this way we prevented undesirable dilution of the analyte by a carrier effect due to some of the constituents present in mixtures of synthetic ampholytes.

The choice of the mobile phase for the chromatographic separations was made by considering mainly the following requirements: (i) a compatibility of the mobile phase with the electrolyte solutions in ITP; (ii) a minimum peak volume of asulam at the column outlet; and (iii) reproducible retention time of asulam in the analysis of soil extracts. Of the alternatives tested[26], the best results were achieved with the mobile phase given under Experimental.

Under the chromatographic conditions employed, the detection limit for asulam

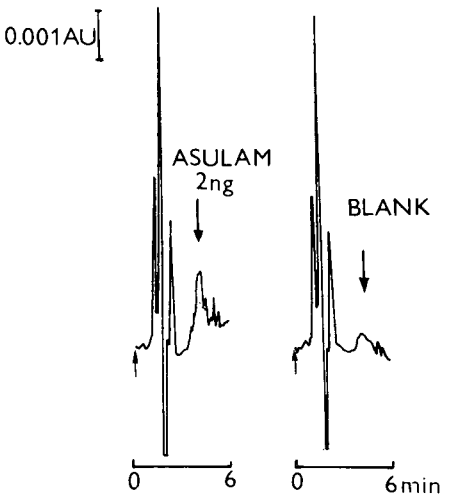

Fig. 2. Chromatograms from the determination of the detection limit of asulam. Asulam was present in a 10^{-2} *M* aqueous solution of sodium sulphate to prevent its losses by adsorption (the blank is a corresponding run with the solution of sodium sulphate). For the composition of the mobile phase and the other working conditions, see Experimental.

was *ca.* 2 ng for a signal-to-noise ratio of 2 (see Fig. 2). This value is considerably higher than that obtained under comparable conditions (the same injection volume, the same detection wavelength and close pH values of the solutions) by ITP. Such a difference may be surprising when a more favourable cell path length of the chromatographic detector is considered (*ca.* 40 times higher than for the ITP detection cell). However, when the dilution of the injected sample during the chromatographic process (*ca.* 15–20 times in this instance) and its concentration during the ITP separation (*ca.* 2000-fold concentration in this instance) are also considered, this difference in the detection limits is obvious. At the same time, these data suggest that here the sequence HPLC–ITP should lead to more favourable analytical results than the opposite order.

Sample clean-up by RP-HPLC with analytical evaluation by ITP

Soil extracts were chosen for this study as they represent matrices of high ionic complexity. Hence the analytical capabilities of the proposed off-line combination of the separation methods could be evaluated for an extreme type of application.

From the characterization of the extraction procedures (see Experimental), it is clear that the methanolic extracts contained very small amounts of the matrix constituents. Chromatograms obtained from their analyses gave the UV profiles shown in Fig. 3. A comparison of the blank run (an extract corresponding to 15 mg of soil sample was injected) with the run in which the extract was spiked with asulam shows that the analyte was eluted on a strongly tailing peak of co-extracted matrix constituents. Further attempts to optimize the chromatographic conditions did not lead to an improvement in the resolution of the analyte from the matrix constituents[26].

An isotachopherogram from the analysis of the same extract as in the blank chromatographic run (Fig. 3, right) shows (Fig. 4) that many of the UV-absorbing

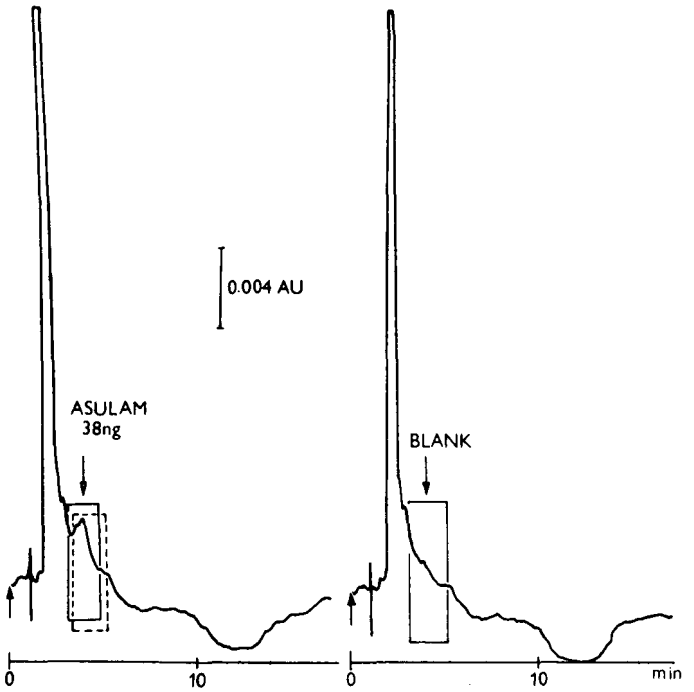

Fig. 3. Chromatograms from the analyses of a methanolic soil extract (right) and the extract spiked with asulam (left). Boxes on the recorder traces mark the collected fractions. For further details, see text.

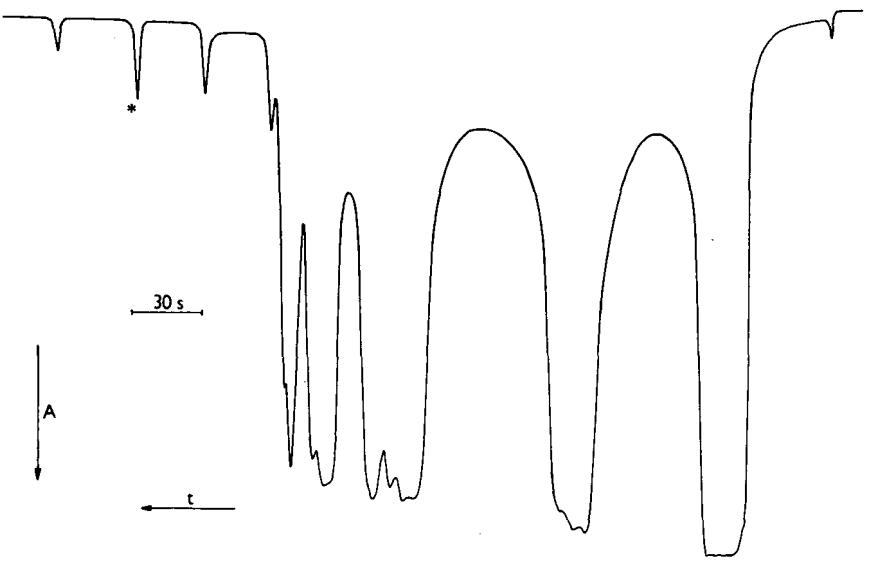

Fig. 4. Isotachopherogram from the analysis of a methanolic soil extract. The same sample as in the blank run in Fig. 3 was taken for the analysis. The same spacing constituents as in Fig. 1 were added to the sample. For the driving currents and the composition of the electrolyte system, see Table I. Symbols as in Fig. 1.

constituents present in this sample are relatively strong acids. Although only a very small part of the matrix constituents was present in the migration position of the analyte (marked with an asterisk in Fig. 4), their contribution to the positive systematic analytical error was equivalent to *ca.* 0.2 ppm of the analyte.

The isotachopherograms in Fig. 5 were obtained from analyses of the corresponding HPLC fractions of the same extract. The isotachopherogram at the top is a complete trace from the photometric detector as obtained from the analysis of the extract containing 0.25 ppm of the analyte (this amount could not be detected in HPLC). To illustrate the contributions of the electrolyte solutions and matrix constituents to the determination of the analyte, only the peaks in its migration position are shown in the box. These peaks clearly show that the impurities from the electrolyte solutions (subtractable from the results of the analyses) represented the main contribution to the positive systematic error in the quantification. Only a 3% contribution of the detected systematic error (equivalent to 0.008 ppm of the analyte) could be ascribed to the matrix constituents. The considerable improvement in this performance parameter in comparison with direct ITP analysis (see above) demon-

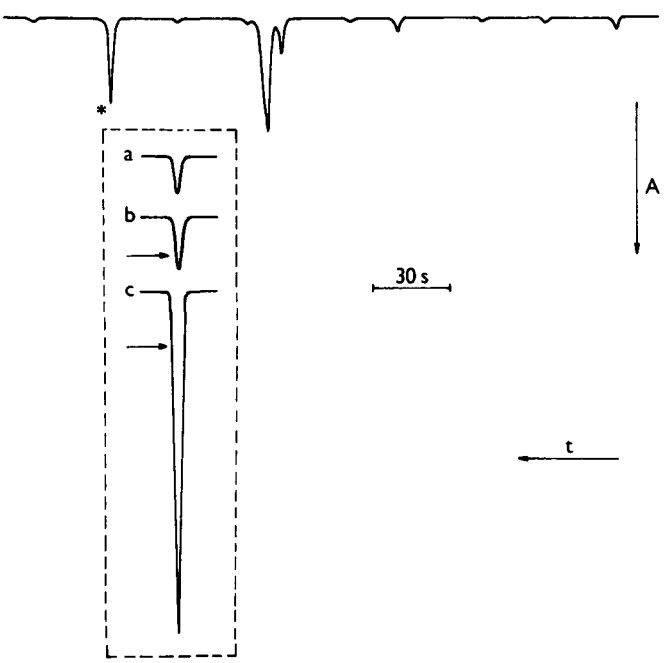

Fig. 5. Isotachopherograms from the analyses of a methanolic soil extract after HPLC clean-up. The same sample as in Figs. 3 and 4 and spiked with asulam at 0.25 ppm was taken for the analysis. The complete upper trace from the photometric detector was obtained in the analysis of fraction of the extract containing asulam (marked with an asterisk). (a) Peak at the migration position of asulam as obtained in a blank run (a mixture of discrete spacers was injected); (b) analysis of a fraction of the unspiked methanolic extract with added discrete spacers (the arrow indicates the peak height corresponding to the impurities in the electrolyte system); (c) as b except that the extract contained asulam (0.25 ppm) (the arrow indicates the peak height that corresponds to the total contribution of the electrolyte solutions and matrix constituents). Isotachopherograms a, b and c were registered with the detector set at a 5-fold higher sensitivity in comparison with the upper trace.

strates the powerful clean-up efficiency of HPLC. As in comparison with the situation shown in Fig. 4 there were almost no UV-absorbing constituents migrating in front of the analyte, this suggest that the separating conditions in both methods were dissimilar (non-correlated).

The sample fractions trapped by HPLC were three times wider relative to the baseline width of the asulam peak to guarantee its quantitative recovery also with fluctuations in the retention times of the separands. However, in this particular instance, it was the widest fraction that could be collected as only a 30-s shift of the start of the fraction collection to shorter retention times (solid-line box on the left-hand chromatogram in Fig. 3) led to a 5-fold increase in the systematic errors of quantitation (a systematic error equivalent to 0.04 ppm of asulam) due to the sample matrix. This is understandable when general requirements concerning multi-dimensional separations in the column-coupling systems are considered[28].

Interactions of pesticides with both inorganic and organic soil constituents are well known[29,30]. Therefore, in such instances more efficient extraction procedures are required. Extraction with borate buffer (see Experimental) belongs to this group of soil extraction procedures. Although in this work there were no reasons to expect problems due to the adsorption of asulam by soil constituents[31], we tested the proposed combination of the separation methods also for such an extreme matrix. From the data characterizing the extraction procedures employed (see Experimental) it is apparent that the borate extract contained a 360-fold excess of soil constituents relative to the methanolic extract. This excess can be ascribed mainly to the extraction of humic acids by the borate buffer solution. Humic acids extracted in this way have pK_a values in the range 1–6 with a maximum occurring close to $pK_a = 4$[32]. Therefore, when these constituents accompany asulam ($pK_a = 4.82$) in the extract, it is apparent that its trace determination by either HPLC or ITP alone would be very tedious.

A typical chromatogram from the analysis of a borate soil extract (pretreated by the SPE procedure as described under Experimental) is given in Fig. 6. From magnified part of the chromatograms at the position of the analyte circled in Fig. 6, it is apparent that its detection in the pretreated borate extract is cumbersome at trace concentrations. The same sample could be analysed by ITP under the working conditions employed only after an appropriate dilution, otherwise high column overloading occurred. In the analysis of the diluted extract, however, we obtained considerably lower systematic errors in the determination of asulam in comparison with the results reported previously[15]. This can be ascribed in part to the improved detection limit (see above) and also to the partial clean-up achieved on the disposable columns. On the other hand, by using HPLC for the isolation of fractions of interest a "clean" sample for the ITP analysis was easily obtained as in this way disturbing inorganic (*ca.* 10^5 excess relative to asulam) and organic constituents (*ca.* 10^6 excess relative to asulam, as estimated from the characterization of the extract) were removed to a substantial extent.

The isotachopherograms in Fig. 7 were obtained from ITP analyses of the borate soil extract after the HPLC clean-up. The complete isotachopherogram from the photometric detector, shown at the top, was obtained in the analysis of a soil sample fortified with the analyte at 12 ppb[a]. The contributions of the electrolyte solutions and

[a] Throughout this article the American billion (10^9) is meant.

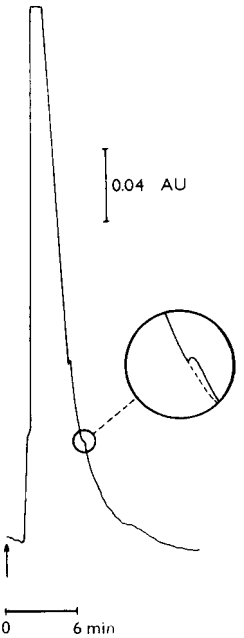

Fig. 6. Chromatogram from the analysis of a borate soil extract spiked with asulam at 0.25 ppm. The injected extract corresponded to 312.5 mg of soil and in the run with the spiked sample it contained 52 ng of asulam. The difference in the chromatograms for the spiked (solid line) and unspiked (dashed line) extracts is shown in the circle. For further details, see text.

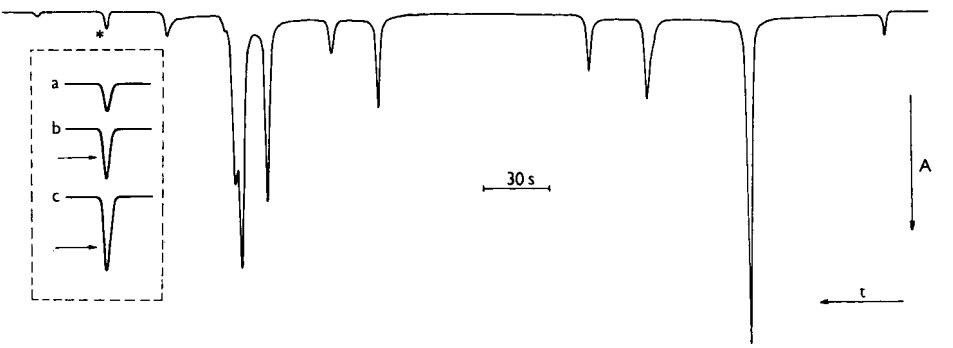

Fig. 7. Isotachopherograms from the analyses of borate soil extracts after HPLC clean-up. The injected parts of the fractions of soil extracts corresponded to 7.8 mg of soil and in the run with the spiked sample 95 pg of asulam was also present. The upper trace was obtained from the photometric detector in the analysis of the fraction containing 12 ppb of asulam (marked with an asterisk). (a) Peak at the migration position of the analyte in a blank run (only a mixture of the discrete spacing constituents was injected); (b) analysis of the fraction from the unspiked extract (the arrow indicates the peak height corresponding to the electrolyte impurities); (c) as b except that the extract was spiked with 12 ppb of asulam (the arrow indicates the peak height corresponding to the sum of comigrating impurities from the extract and electrolyte solution). Isotachopherograms a, b and c were registered at a 5-times higher amplification of the photometric detector in comparison with the upper trace.

the soil matrix to its determination are illustrated in the box. In this instance the matrix constituents migrating at the position of the analyte represented a positive systematic error of 12 ppb. This value is close to that achieved with the methanolic extract and clearly indicates that a very efficient sample clean-up was achieved by HPLC also in this instance. Thus, in this instance (12 ppb of the analyte), a *ca.* 100% systematic error was involved. When the complexity of the matrix is considered, in many instances this is a tolerable bias of the determination.

CONCLUSIONS

The results clearly show that RP-HPLC can be a very efficient sample preparation method in ITP analysis. In our experiments its use was straightforward as the sample clean-up was a key requirement and an inherent sample dilution during the chromatographic separation was compensated for in the final analytical step by the concentrating power of ITP. In this respect, the low detection limit achieved in the ITP analysis owing to the use of selective detection in the spike mode of quantification was also advantageous. However, our previous experience in the use of HPLC for sample preparation in ITP[33] indicated that in the evaluation of the analysis using a high-resolution universal detector the chromatographic dilution of the constituents of interest may require injection volumes as large as several millilitres. Although sample injection devices suitable for such volumes have been developed for ITP[23], the presence of ionogenic constituents in the HPLC mobile phase at concentrations as low as 10^{-3}–10^{-4} M may require unrealistic load capacities of the ITP columns. These facts indicate that when this sequence of the separation methods is intended for trace analytical work, the use of selective detectors in the ITP step may be essential.

As in this work a very simple configuration of the chromatographic instrument was satisfactory for the trace analysis experiments with matrices of extreme ionic complexity, it appears that the use of HPLC for sample preparation in ITP is an economic solution in general. In this respect, it should be stressed that by using the ITP equipment with the column-coupling configuration of the separation unit there are additional possibilities for further sample clean-up and/or for concentration of the analyte.

The time needed for a complete analysis (not including the soil extractions) was *ca.* 35 min or less. In comparison with previous procedures[15] this represents at least a 50% reduction in the analysis time. It appears that a short analysis time is a typical feature of the sequence HPLC–ITP because, *e.g.*, removal of inorganic salts from the samples can considerably reduce the time requirements for the ITP analysis.

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Note

Utilization of capillary isotachophoresis in the determination of organic acids in food

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The contents of organic acids in various food products have been determined by traditional analytical methods[1], but such analyses are time consuming and suffer from errors due to the large number of operations necessary for the isolation and determination of individual components. In addition, the presence of other acids and/or materials can interfere and cause additional errors. Hence modern separation methods have been increasingly used[2–5].

The detection of individual acids in various food products is now carried out mostly by chromatographic methods, gas chromatography[6,7] and high-performance liquid chromatography[8,9] being the most widely used. However, the complete chromatographic separation of organic acids is not easily achieved, and the methods are sometimes time consuming and involve complex sample preparation organic acids in aqueous alcohols have been determined using zone electrophoresis[10,11].

Capillary isotachophoresis has been found suitable for the identification and determination of organic acids in foods[12–26]. Its main advantages are simple sample preparation and short analysis times. Separation can be performed in both aqueous and non-aqueous solutions [27,28].

We have used capillary isotachophoresis in the study of the formation of organic acids and phosphoric acid during lactic fermentation of cabbage and of changes in organic acid contents during the maturation of red wine.

EXPERIMENTAL

We used a CS ZKI 001 Isotachophoretic Analyser (Spišská N. Ves, Czechoslovakia) with a conductivity detector and a TZ 4200 double-line recorder (Laboratorní přístroje, Prague, Czechoslovakia), and also several electrolytic systems as shown in Table I. Samples diluted 1:50 with water were injected into the column using the four-way valve of the instrument. The duration of the analysis was 20–30 min, depending on the electrolytes used. Organic acids were identified by comparison of the isotachophoretic zones of the analytes in the samples and in standard solutions. Quantitative analysis was performed by calibration.

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TABLE I

COMPOSITION OF ELECTROLYTES AND CONDITIONS OF SEPARATION

Concentration of leading electrolyte (hydrochloric acid) (M)	10^{-2}	10^{-2}	10^{-2}
Counter ion	β-Alanine	ε-Aminocaproic acid	
pH	3.8	4.5	5.2
Additive polyvinylpyrrolidone (PVP) (%)	0.1	0.1	0.1
Terminating electrolyte	Caproic acid	Caproic acid	Caproic acid
Concentration (M)	$5 \cdot 10^{-3}$	10^{-2}	10^{-2}
Current of 200 μA in the preseparation column			
Current of 40 μA in the analytical column			

RESULTS AND DISCUSSION

The process of cabbage fermentation was followed by the determination of organic acids in the liquid phase. Lactic, acetic, phosphoric and ascorbic acid were determined after 4, 5, 6, 7, 10 and 11 days of fermentation. Fig. 1 shows the increase in organic acid contents. After 11 days of fermentation, the following acid contents were found in the liquid portion of fermented cabbage: ascorbic acid 0.16, lactic acid 6.13, acetic acid 1.52 and phosphoric acid 0.14 g l^{-1}. Lactic and acetic acid are the main

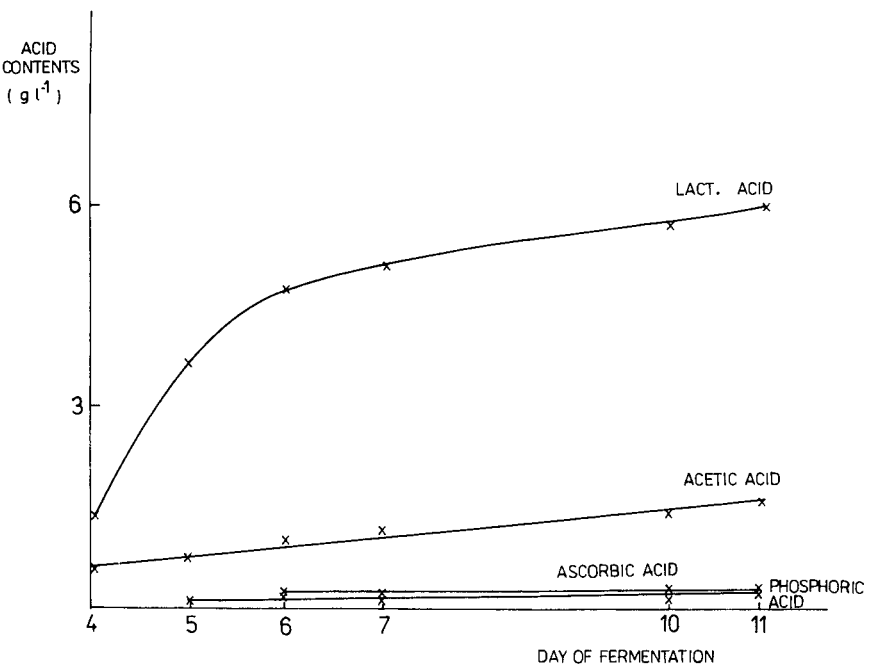

Fig. 1. Organic acids in fermented cabbage. LACT. = lactic.

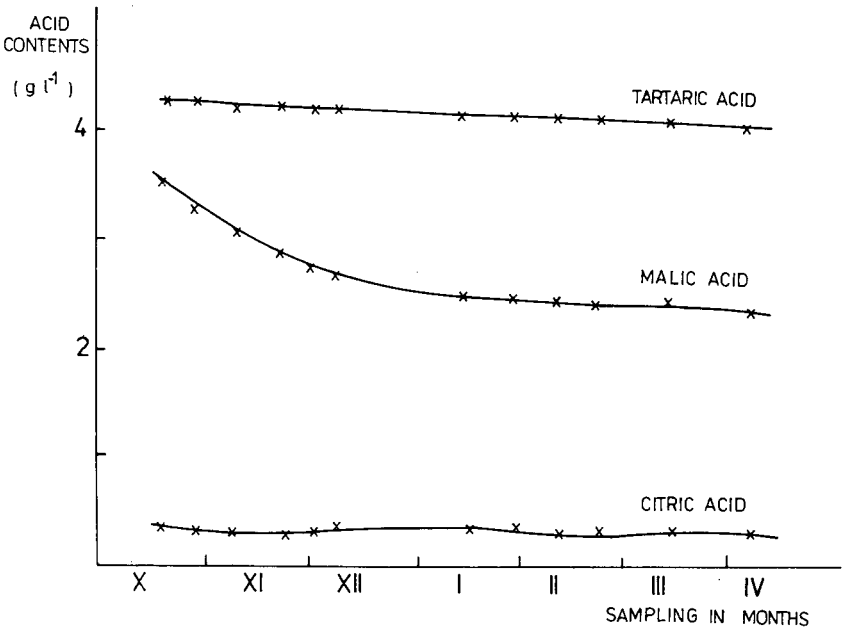

Fig. 2. Changes in tartaric, malic and citric acid contents in Svätovavrinecké red wine.

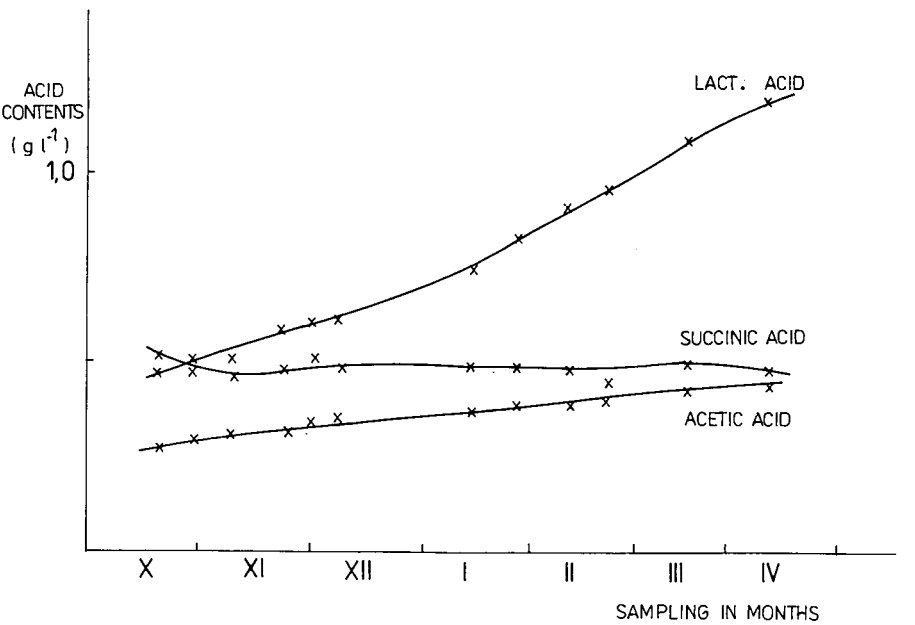

Fig. 3. Changes in lactic, succinic and acetic acid contents in Svätovavrinecké red wine.

products of the lactic fermentation process and are a significant parameter for the quality of fermented cabbage. By comparing the results obtained after 11 days of fermentation and those obtained by Klein[29] and Hamtschek[30], a further increase in the acid contents with prolonged fermentation could be assumed, with citric acid being formed in the final stage.

We also studied red wine formation from Svätovavrinecké (Saint Laures) grape mash. The wine was decanted from the dregs as the residual sugar content decreased to 4 g l^{-1}. The decanted wine was stored in glass containers and the changes in the contents of tartaric, malic, citric, lactic, succinic and acetic acid were followed by isotachophoresis. The organic acids formed influence the characteristic a stringent taste of the wine. Figs. 2 and 3 show the changes in the acid contents in the wine during maturation from October to April.

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Computer-assisted choice of electrolyte systems and spacing constituents for two-dimensional capillary isotachophoresis

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ABSTRACT

Equations enabling interferences due to sample matrix constituents in two-dimensional isotachophoretic analysis using the spike method to be estimated were derived. A computer program and a procedure based on these equations were developed for searching for optimum and less time consuming electrolyte systems and spacing constituents. The procedure, intended mainly for the trace analysis of the constituents present in complex ionic matrices, was examined in experiments with benzoate as a trace analyte. A model mixture of 50 arbitrarily chosen anionically migrating UV light-absorbing constituents simulated the sample matrix. A reasonable agreement in ordering the electrolyte systems according to interfering contributions of the sample matrix was obtained between the experimental and calculated data. Some discrepancies found in the examination of the proposed procedure are discussed.

INTRODUCTION

Column-coupling capillary isotachophoresis (CC-ITP), as first described by Everaerts *et al.*[1], has several advantages in the analysis of complex ionic mixtures when both a high performance index of the separation compartment[2] and high resolution rates[3] are desired. In addition, this technique enables two-dimensional (2D) ITP separations to be performed when some general requirements concerning 2D separations in the column-coupling systems[4] are met. It can easily be deduced that for ITP they can be formulated in the following way: (i) the sample constituents are subjected to two largely independent separation steps, *i.e.*, the leading electrolytes in the coupled columns should be chosen among those characterized by low similarities[5];

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and (ii) the separation achieved in the first dimension is not nullified by the formation of mixed zones in the second separation step.

The latter requirement clearly implies that in CC-ITP maximum analytical benefit, *i.e.*, a true 2D separation, can be achieved when apart from the analyte only a minimum number of sample constituents are transferred from the first column for a final separation in the second column. It also suggests that a tandem arrangement of independently refillable columns[6] is, in general, inconvenient for 2D ITP separations.

In ITP, selective detectors often provide considerably lower detection limits than high-resolution universal detectors, especially when the spike[7–9] and/or steady-state mixed zone[10] methods are employed. Therefore, 2D ITP separations combined with the selective detection of the analytes by one of these methods appear promising for trace analytical work, potentially with no or at least minimized sample preparation. In such a combination, appropriate discrete spacing constituents added to the sample can be effective in achieving the following goals[11]: (i) a well defined transfer of the sample fraction of analytical interest from the first separation step for a final separation in the second separation step; and (ii) favourable detection conditions for the analyte in the second separation step.

From the practical point of view, a choice of the leading electrolytes and spacing constituents giving a minimum bias in the detection and/or determination of the analyte can be both time and labour consuming when performed only experimentally. When the qualitative sample composition is known, computer simulation provides a means of reducing this problem[12–14]. Unfortunately, in the analysis of trace constituents present in complex matrices the sample composition is known only seldom and, therefore, this straightforward approach has limited applicability in these instances.

The aim of this work was to elaborate a procedure that could facilitate a search for suitable combinations of the leading electrolytes and spacing constituents in 2D ITP when the analytical evaluation is performed by the spike method from the response of the selective detector. The procedure described here is based on relationships that allow a comparison of various electrolyte systems and discrete spacers via parameters characterizing (potential) interferences in the determination of the analyte due to matrix constituents. A computer program providing numerical values of these parameters was written. The proposed procedure was applied to the determination of benzoate present at a $5 \cdot 10^{-6}$ mol/l concentration in a mixture containing 50 anionic constituents representing the sample matrix.

THEORETICAL

Interfering constituents in 2D ITP analysis of complex mixtures

2D ITP separation with selective detection of the analyte in the second separation dimension (2nd D) is illustrated schematically in Fig. 1. From basic principles of ITP[15], it is clear that the migration configuration shown in Fig. 1 requires that in the first separation dimension (1st D) the following conditions are fulfilled:

$$ {}_1\bar{m}_{X,A} < {}_1\bar{m}_{A,A} \tag{1a}$$

and

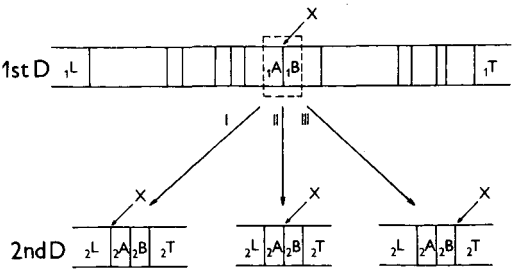

Fig. 1. Scheme of 2D ITP analysis in the column-coupling instrument with selective detection of the analyte migrating in the interzonal boundary layer between the zones of spacers A and B. The dashed box indicates that part of the sample fraction transferred for a final separation in the 2nd D. I, II, III = possible migration configuration of the analyte (X) under the working conditions employed in the 2nd D when the formation of mixed zones and/or a zone-electrophoretic migration of the analyte do not occur. L, T = leading and terminating constituents, respectively; 1, 2 = indices of the separation dimensions.

$$ {}_1\bar{m}_{X,B} > {}_1\bar{m}_{B,B} \tag{1b} $$

where the subscript 1 identifies the 1st D, the first subscripts on the right relate to the constituents and the second subscripts on the right identify the zones.

As we consider the complex ionic nature of the sample, it can be expected that the analyte in its migration position is accompanied by some of the sample constituents. In our particular case, under the conditions employed in the 1st D, matrix constituents (Z) will interfere, giving a response to the detector and meeting the conditions

$$ {}_1\bar{m}_{Z,A} \leqslant {}_1\bar{m}_{A,A} \tag{2a} $$

and

$$ {}_1\bar{m}_{Z,B} \geqslant {}_1\bar{m}_{B,B} \tag{2b} $$

where the sign of equality indicates that interferents present within the zones of the spacers are also included (the steady-state mixed zones). For the sake of simplicity we assume in this work that the interferents are strong and monovalent, weakly ionic constituents and that only acid–base equilibria are involved in the separation. Then for the effective mobilities of the interfering constituents we can write

$$ {}_1\bar{m}_{Z,i} = {}_1\alpha_{Z,i}\,{}_1m_Z \tag{3} $$

where ${}_1\alpha_{Z,i}$ is a molar fraction (degree of dissociation) of the charged ionic form of a given constituent in the ith zone. The molar fraction and the corresponding dissociation constant (K_Z) are related via the Henderson–Hasselbach equation

$$ {}_1\alpha_{Z,i} = K_Z/({}_1[H]_i + K_Z) \tag{4} $$

where ${}_1[H]_i$ is the concentration of H^+ ions in the ith zone. Eqns. 3 and 4 enable the conditions (2a) and (2b) to be modified into the following form:

$$ {}_1m_Z \leqslant {}_1\bar{m}_{A,A}(1 + 10^{pK_Z - {}_1pH_A}) \tag{5a} $$

and

$$ {}_1m_Z \geqslant {}_1\bar{m}_{B,B}(1 + 10^{pK_Z - {}_1pH_B}) \tag{5b} $$

where ${}_1pH_A$ and ${}_1pH_B$ are the pH values in the zones of spacers in the 1st D.

The ionic mobilities and pK values of both the analyte and spacing constituents and the composition of the leading electrolyte used in the 2nd D determine the actual migration configuration in this separation step. When neither zone electrophoretic migration of the analyte nor formation of a mixed zone of the analyte with one of the constituents occurs, then in this instance one of the migration configurations shown in Fig. 1 will be achieved in the 2nd D. For the interfering constituents analogous relationships to those for the 1st D are valid. Hence, for the configuration discussed further (III in Fig. 1), we can write the conditions

$$ {}_2m_Z \leqslant {}_2\bar{m}_{B,B}(1 + 10^{pK_Z - {}_2pH_B}) \tag{6a} $$

and

$$ {}_2m_Z \geqslant {}_2\bar{m}_{T,T}(1 + 10^{pK_Z - {}_2pH_T}) \tag{6b} $$

where the subscript 2 identifies the 2nd D.

Clean-up efficiency in 2D ITP

Characteristic parameters for ITP migrating zones can be calculated, *e.g.*, as elaborated by Beckers and co-workers[15,16]. The data obtained permit the use of eqns. 5 and 6 to calculate plots defining regions of interfering constituents (in coordinates of ionic mobility *vs.* pK value) for both separation steps. When a uniform distribution of the interfering constituents within given ranges of ionic mobilities and pK values is assumed, then the areas of the regions of interferents can be interpreted in the following way (see also Fig. 2): the area ${}_1A$ is a measure of interfering matrix constituents unseparable from the analyte under the conditions employed in the 1st D (the area ${}_2A$ is an analogous measure for the 2nd D); an overlap of the areas ${}_1A$ and ${}_2A$ (${}_{1-2}A$ in Fig. 2) serves as a measure of the constituents unseparable from the analyte with a given combination of the leading electrolyte and spacing constituents; the unoverlapped part of the area ${}_1A$ corresponds to that part of interfering constituents which is separated from the analyte under the conditions employed in the 2nd D; and the unoverlapped part of the area ${}_2A$ represents that part of the constituents which interfere under the conditions employed in the 2nd D but which cannot be present in the sample fraction transferred into the 2nd D.

From this interpretation, it is apparent that the ratio ${}_{1-2}A/{}_1A$ is a measure of a clean-up efficiency of the 2nd D for a given sequence of the leading electrolytes and for the spacing constituents employed. As the clean-up efficiency of the 2nd D (ΔS_x) is zero when the same leading electrolytes and spacing constituents are used in both separation steps, it is reasonable to define this parameter via the equation

$$ \Delta S_x = 1 - {}_{1-2}A/{}_1A \tag{7} $$

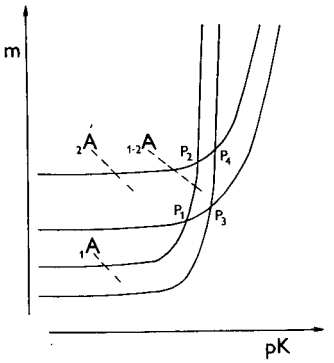

Fig. 2. Schematic illustration of plots defining regions of interfering constituents in the coordinates of ionic mobility (m) *vs.* pK value. $_1A$ = region (area) of interfering constituents accompanying the analyte in the 1st D (these constituents are transferred with the analyte for a final separation in the 2nd D). Solid lines defining this region correspond to the interfering constituents migrating in the zones of spacing constituents. $_2A$ = region (area) of interfering constituents accompanying the analyte under the working conditions employed in the 2nd D. Solid lines have an analogous meaning to those in the 1st D. $_{1-2}A$ = region (area) corresponding to the matrix constituents unseparable from the analyte in a given combination of the electrolyte systems employed in both dimensions. P_1–P_4 = points of intersection of the solid lines defining the regions $_1A$ and $_2A$.

Despite the above simplifying assumptions, eqn. 7 may serve as a realistic estimate of the clean-up efficiency of the 2nd D for matrices with characteristic "continuous" mobility profiles[17–19].

It is apparent that by using the same leading electrolyte in both columns with the same spacing constituents, $_{1-2}A = {}_1A = {}_2A$ and $\Delta S_x = 0$. The value $\Delta S_x = 1$, *i.e.*, a maximum clean-up efficiency in the 2nd D, is meaningless as it requires that $_{1-2}A = 0$. In other words, in this instance the areas $_1A$ and $_2A$ do not overlap and, therefore, the analyte cannot migrate in the desired position in the 2nd D. Hence only the values $\Delta S_x < 1$ have a practical meaning.

Comparison of various combinations of leading electrolytes and spacing constituents

In 2D ITP it is desirable to achieve a high clean-up efficiency in the 2nd D($\Delta S_x \rightarrow 1$). On the other hand, this need not to correspond to an optimum combination of the leading electrolytes and/or spacing constituents because it can be due to a high value of $_1A$ (a wide fraction transferred for a final separation in the 2nd D). Therefore, a comparison of various combinations of the electrolyte systems and spacing constituents is more convenient via their $_{1-2}A$ values since this parameter may serve as an absolute measure of interfering constituents unresolved from the analyte.

It is apparent that an optimum combination of the leading electrolytes and discrete spacers is characterized by a minimum value of $_{1-2}A$ (ideally, $_{1-2}A \rightarrow 0$). Once such a combination has been found, it is necessary to select an optimum sequence of the leading electrolytes in the separation steps. From general requirements (see Introduction) it can be seen that it is desirable to transfer a minimum of the matrix constituents from the first column for a final separation in the second column. This is fulfilled when the leading electrolyte with associated spacers having a lower area defining the interfering constituents is used in the first column. Moreover, such

a criterion is favourable when we consider also the volumes of the columns advantageously used in CC-ITP[1,20].

Equations for calculations of parameters for pre-experimental choice of leading electrolytes and spacing constituents

Eqns. 5 and 6 enable one to calculate and plot interfering constituents as shown in Fig. 2. Numerical values of the areas ${}_{1-2}A$, ${}_{1}A$ and ${}_{2}A$, important for the choice of optimum working conditions, can be obtained by solving the following integrals within reasonable chosen limits of pK values (0–11 in this instance) and within limits defined by the pK values corresponding to the points of intersection ($P_1 - P_4$ in Fig. 2):

$$ {}_{1}A = \int_{0}^{11} ({}_{1}\bar{m}_{A,A} \cdot 10^{pK_Z - {}_{1}pH_A} + {}_{1}\bar{m}_{A,A})\, dpK_Z - \int_{0}^{11} ({}_{1}\bar{m}_{B,B} \cdot 10^{pK_Z - {}_{1}pH_B} + {}_{1}\bar{m}_{B,B})\, dpK_Z \quad (8) $$

$$ {}_{2}A = \int_{0}^{11} ({}_{2}\bar{m}_{B,B} \cdot 10^{pK_Z - {}_{2}pH_B} + {}_{2}\bar{m}_{B,B})\, dpK_Z - \int_{0}^{11} ({}_{2}\bar{m}_{T,T} \cdot 10^{pK_Z - {}_{2}pH_T} + {}_{2}\bar{m}_{T,T})\, dpK_Z \quad (9) $$

$$ {}_{1-2}A = \int_{pK'_{Z_1}}^{pK'_{Z_2}} ({}_{1}\bar{m}_{A,A} \cdot 10^{pK_Z - {}_{1}pH_A} + {}_{1}\bar{m}_{A,A})\, dpK_Z - \int_{pK'_{Z_1}}^{pK'_{Z_2}} ({}_{2}\bar{m}_{T,T} \cdot 10^{pK_Z - {}_{2}pH_T} + {}_{2}\bar{m}_{T,T})\, dpK_Z + $$

$$ \int_{pK'_{Z_2}}^{pK'_{Z_3}} ({}_{2}\bar{m}_{B,B} \cdot 10^{pK_Z - {}_{2}pH_B} + {}_{2}\bar{m}_{B,B})\, dpK_Z - \int_{pK'_{Z_2}}^{pK'_{Z_3}} ({}_{2}\bar{m}_{T,T} \cdot 10^{pK_Z - {}_{2}pH_T} + {}_{2}\bar{m}_{T,T})\, dpK_Z + $$

$$ \int_{pK'_{3_1}}^{pK'_{Z_4}} ({}_{1}\bar{m}_{B,B} \cdot 10^{pK_Z - {}_{1}pH_B} + {}_{1}\bar{m}_{B,B})\, dpK_Z - \int_{pK'_{Z_3}}^{pK'_{Z_4}} ({}_{2}\bar{m}_{T,T} \cdot 10^{pK_Z - {}_{2}pH_T} + {}_{2}\bar{m}_{T,T})\, dpK_Z \quad (1 $$

The solutions of the integrals[21] are illustrated for the first integral in eqn. 8

$$ {}_{1}\bar{m}_{A,A} \cdot 10^{-{}_{1}pH_A} \cdot \int 10^{pK_Z}\, dpK_Z + \int {}_{1}\bar{m}_{A,A}\, dpK_Z \quad (11) $$

where the solution is

$$ {}_{1}\bar{m}_{A,A}/(\ln 10) \cdot 10^{pK_Z - {}_{1}pH_A} + {}_{1}\bar{m}_{A,A} pK_Z + C \quad (12) $$

In the case in Fig. 2, for the points of intersection the following conditions are valid:

$$P_1: \quad {}_1\bar{m}_{A,A} \cdot 10^{pK'_{Z_1} - {}_1pH_A} + {}_1\bar{m}_{A,A} = {}_2\bar{m}_{T,T} \cdot 10^{pK'_{Z_1} - {}_2pH_T} + {}_2\bar{m}_{T,T} \tag{13}$$

$$P_2: \quad {}_1\bar{m}_{A,A} \cdot 10^{pK'_{Z_2} - {}_1pH_A} + {}_1\bar{m}_{A,A} = {}_2\bar{m}_{B,B} \cdot 10^{pK'_{Z_2} - {}_2pH_B} + {}_2\bar{m}_{B,B} \tag{14}$$

$$P_3: \quad {}_1\bar{m}_{B,B} \cdot 10^{pK'_{Z_3} - {}_1pH_B} + {}_1\bar{m}_{B,B} = {}_2\bar{m}_{T,T} \cdot 10^{pK'_{Z_3} - {}_2pH_T} + {}_2\bar{m}_{T,T} \tag{15}$$

$$P_4: \quad {}_1\bar{m}_{B,B} \cdot 10^{pK'_{Z_4} - {}_1pH_B} + {}_1\bar{m}_{B,B} = {}_2\bar{m}_{B,B} \cdot 10^{pK'_{Z_4} - {}_2pH_B} + {}_2\bar{m}_{B,B} \tag{16}$$

which provide pK values (pK'_{Z_1}–pK'_{Z_4}) required for the solutions of the integrals in eqn. 10.

EXPERIMENTAL

Instrumentation

A CS isotachophoretic analyser (VVZ PJT, Spišká Nová Ves, Czechoslovakia) was used in the column-coupling configuration of the separation unit. The analytical column of the analyser was provided with an on-column UVD1 photometric detector (VVZ PJT) and the detection wavelength was 254 nm. The signal from this detector was evaluated with an HP 3390A reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The program, written in T-Pascal 1.0 (VÚVT, Žilina, Czechoslovakia), runs on a DATAS-AT computer (DataSystem, Bratislava, Czechoslovakia).

Chemicals

Chemicals used for the preparation of the leading and terminating electrolytes were obtained from Sigma (St. Louis, MO, U.S.A.), Serva (Heidelberg, F.R.G.), Reanal (Budapest, Hungary) and Lachema (Brno, Czechoslovakia). They were purified by current methods[22]. Water supplied by a Rodem 1 demineralization unit (OPP, Tišnov, Czechoslovakia) and further purified on Amberlite MB-1 mixed-bed ion exchanger (BDH, Poole, U.K.) was used for the preparation of the solutions.

Hydroxyethylcellulose 4000 (Serva) was added to the leading electrolytes as an anticonvective additive. Its stock solutions were purified on Amberlite MB-1 mixed-bed ion exchanger.

RESULTS AND DISCUSSION

Description of the calculations

Calculations of the parameters required for a pre-experimental choice of the working conditions (see Theoretical) were carried out in the following steps: calculations of characteristic parameters of the zones of spacing constituents and the terminating zone in the ITP steady state were carried out with the program ITER[15,16], physico-chemical data published by Hirokawa *et al.*[23] serving as input data; calculations to examine whether the conditions (1) and/or analogous conditions for

the 2nd D are met; by using eqns. 5 and 6, boundaries determining the regions of interfering constituents were calculated (see also Fig. 2); the area of overlap (${}_1{}_{-2}A$) and the areas of the regions of interfering constituents (${}_1A$ and ${}_2A$) were calculated from eqns. 8–10; calculations of the clean-up efficiency of the 2nd D with the aid of eqn. 7.

A detailed description of these calculations is apparent from the algorithm given in Fig. 3. In the calculations we assumed that the interfering constituents have ionic mobilities in the range $0–10^{-3}$ cm^2 V^{-1} s^{-1} while their pK values are 0–11. The results of the calculations and graphical plots analogous to those shown schematically in Fig. 2 could be displayed on a screen and/or printed.

Comparison of calculated and experimental data

In an experimental examination of the proposed procedure, benzoate was chosen as an analyte and a mixture of arbitrarily chosen UV light-absorbing constituents (Table I) migrating anionically under the working conditions studied

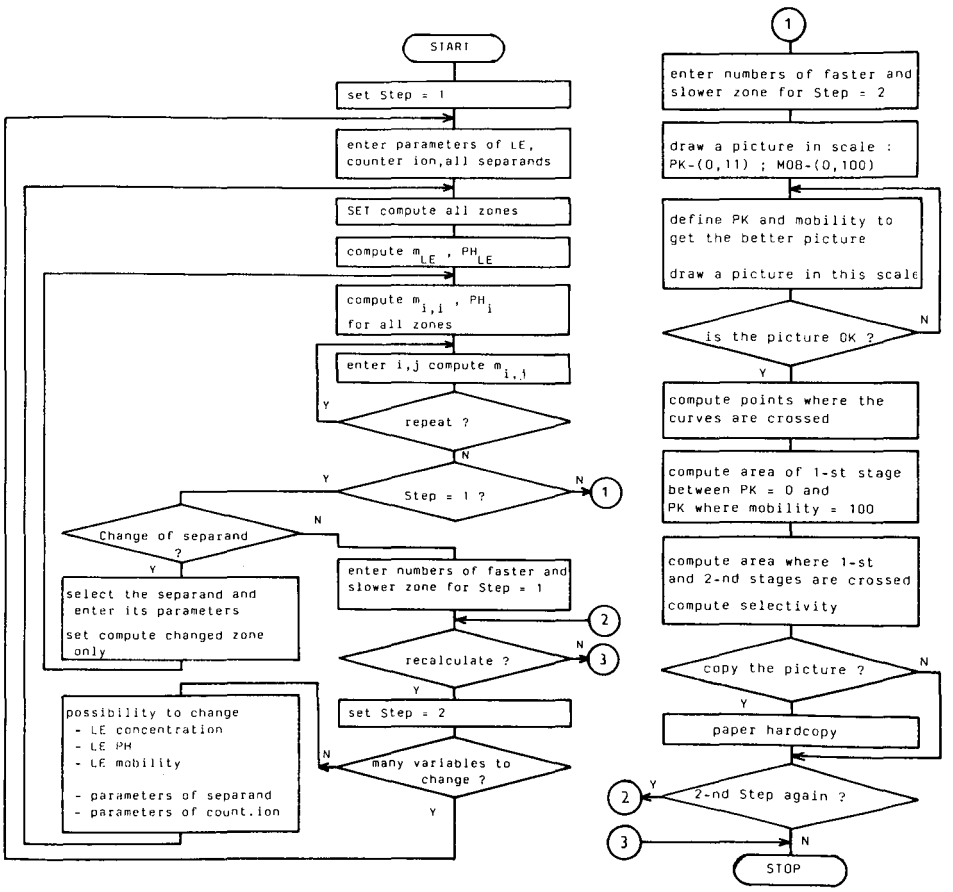

Fig. 3. Algorithm for the calculation of the steady-state parameters of the zones and the parameters characterizing the electrolyte systems and spacing constituents from the point of view of the interferences due to the sample matrix constituents.

TABLE I

COMPOSITION OF MODEL MIXTURE USED AS A SAMPLE MATRIX

Acetylsalicylic, anthranilic, *p*-aminobenzoic, *p*-amino-2-naphthol-4-sulphonic, anthraquinone-2-sulphonic, 2-amino-8-naphthol-3,6-disulphonic, *p*-aminosalicylic, 2-amino-5-naphthol-7-sulphonic chromotropic, cinnamic, 2-amino-5-naphthol-3,6-disulphonic, 4-chlorobenzoic, 2,4-dihydroxybenzoic, fumaric, 5,5-diethylbarbituric, 2,4-dinitrobenzoic, 3,5-dinitrosalicylic, furylacrylic, 3,5-dinitrobenzoic, 4,4′-diaminostilbene-2,2′-disulphonic, β-indolylacetic, 2,5-dichlorobenzenesulphonic, *m*-hydroxybenzoic, hippuric, 2,4,6-trimethylbenzenesulphonic, 2-ketoglutaric, mandelic, maleic, methanilic, *m*-nitrobenzoic, *p*-nitrobenzoic, 3-nitrophthalic, nicotinic, naphthylacetic, 2-naphthoxyacetic, 4-nitrophthalic, 3-nitrobenzenesulphonic, phenylanthranilic, picric, pyrogallolcarbonic, phenylphosphoric, phthalic, sulphosalicylic, sorbic, sulphanilic, salicylic, 2,4,6-trinitrobenzoic and *p*-toluenesulphonic acids, 2,6-dinitrophenol and 2,4-dinitrophenol

served as a sample matrix. The concentrations of the matrix constituents in the injected samples (valve injection with a 30-μl sample loop) were *ca.* 0.2 ppm to achieve a linear response of the photometric detector for the constituents present in the migration position of benzoate in all experiments.

The isotachopherograms in Fig. 4 provide detection profiles of the sample matrix in the operational systems in which the separations were performed, mainly according to pK values (pH 3.2) and according to ionic mobilities (pH 4.7). With respect to some inherent limitations of the separation unit employed (the same terminating constituents for both columns), these systems were taken as lower (pH 3.2) and upper (pH 4.7) pH limits in this work.

When the columns were filled with the same leading electrolytes ("single" column mode of analysis) and only that part of the sample containing the analyte with the adjacent parts of the zones of spacing constituents were transferred to the second column for detection, we obtained isotachopherograms (Fig. 5) that provided the following information: (i) the total amount of photometrically detectable impurities (measured in integrator counts) present in the migration position of benzoate and originating from the electrolyte system used (Fig. 5a); when desired this contribution to the systematic error in the quantification of the analyte could be subtracted as a blank value; (ii) the total amount of the matrix constituents present in the migration position of the analyte and detectable by the photometric detector [measured as in (i)]; its value (Fig. 5b) is related to the areas ${}_1A$ and/or ${}_2A$ as defined under Theoretical; (iii) the response of the detector corresponding to the analyte (the difference between the numbers of counts as measured in Fig. 5c and b); and (iv) characterization of the sample fraction transferred for a final separation in the second column when a given electrolyte system is used in the first column in 2D runs.

Isotachopherograms from the photometric detector shown in Fig. 6 were obtained under the conditions with given sequences of the leading electrolytes. The data provided by the detector (measured in the above way) were taken as experimental estimates of contributions of the electrolyte systems (Fig. 6a) and matrix constituents (the difference between Fig. 6b and a) to the quantification of the analyte. It is clear that the runs with the sample matrix (Fig. 6b) provided the experimental data corresponding to the areas ${}_{1-2}A$ (Fig. 2) calculated as described above.

The data obtained from the described experiments and those from the calculations are summarized in Table III. A direct comparison of the experimental and

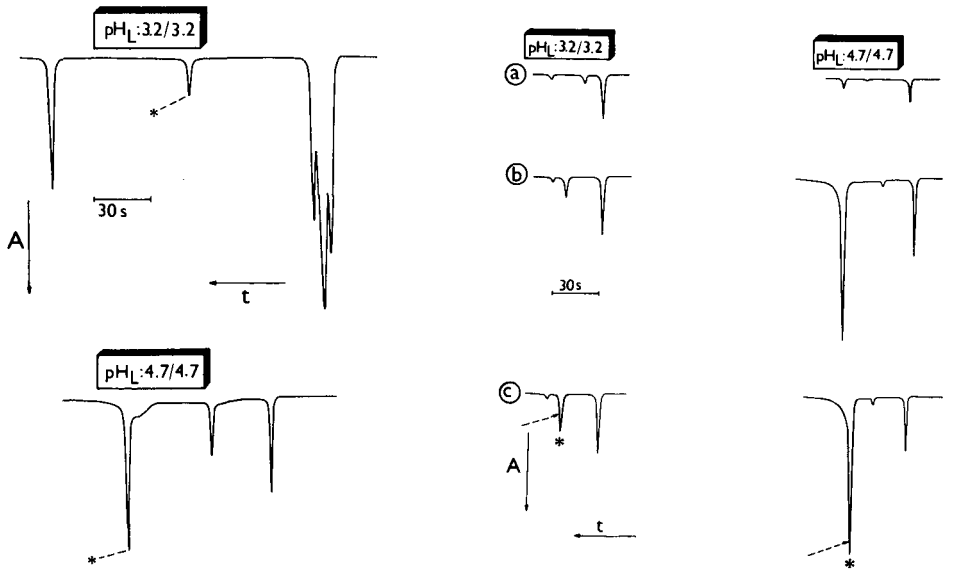

Fig. 4. Isotachopherograms from the analyses of a model mixture representing the sample matrix (see Table I and the text). Only the traces from the photometric detector in the second column are given. Asterisks mark the migration positions of benzoate. Succinate and glutarate served as the discrete spacers in these experiments. A,t = increasing light absorption and time, respectively. The driving currents were 200 and 45 μA in the first and second columns, respectively. The operational systems (Table II) are indicated via their pH_L values.

Fig. 5. Contributions of the interferences from (a) the electrolyte system and (b) the sample matrix to (c) the quantification of benzoate ($5 \cdot 10^{-6}$ mol/l concentration in the injected sample). Both columns were filled with identical leading electrolytes (see the corresponding data in this figure and Table II) and only that part of the sample containing the analyte and adjacent regions of the zones of spacers (succinate and glutarate) were transferred into the second column for detection. Arrows indicate peak heights corresponding to the interfering constituents in the migration position of benzoate (marked with asterisks). For the driving currents and the meaning of the remainder of the symbols, see Fig. 4. The operational systems (Table II) are indicated via their pH_L values.

calculated data of primary interest ($_{1-2}A$ and $_{1-2}A^*$ in Table III) was almost impossible as they were obtained in different physical units mutually convertible only with difficulty. Therefore, the practical utility of the choice of the electrolyte systems for 2D ITP via the calculations based on our simple model could be evaluated only qualitatively. As our main aim was to develop a procedure to simplify the search for optimum working conditions, this is an acceptable way. In the evaluation, the electrolyte systems considered in the investigation were ordered according to increasing $_{1-2}A$ values, *i.e.*, in the order of expected bias in the determination of the analyte. From the tabulated data the following order of the electrolyte systems was obtained: II < III < IV < I. It should be noted that the $_{1-2}A$ values for systems II and III are *a priori* identical (different sequences of the same pair of leading electrolytes). Therefore, $_1A$ values were decisive in their ordering (see Theoretical). The same electrolyte systems ordered on the basis of experimental data ($_{1-2}A^*$ values in Table III) gave the following order: II < III < I < IV. A comparison of the ordered

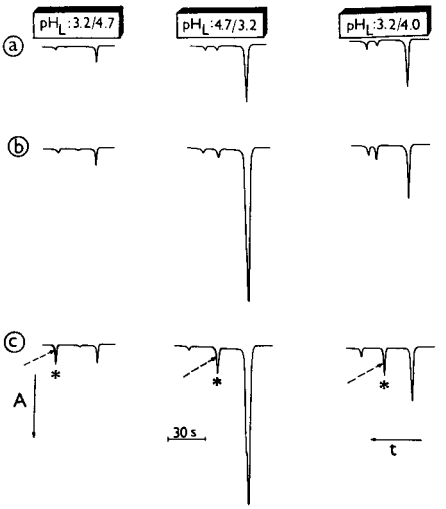

Fig. 6. Contributions of the interferences from (a) the electrolyte system and (b) the sample matrix to (c) the quantification of benzoate in the 2D mode of the ITP analysis for various sequences of the leading electrolytes (see the data in this figure and Table II). Arrows indicate peak heights corresponding to the interfering constituents migrating in the same position as benzoate (marked with asterisks) under given working conditions. The same samples as in Fig. 5 were injected in these experiments. For the driving currents and the meaning of the remainder of the symbols, see Fig. 4.

electrolyte systems suggest that the calculated parameters enabled an optimum electrolyte system to be found from the considered alternatives (after the spacing constituents had been chosen). It is apparent that the choice of the spacing constituents can be optimized in an analogous way once the electrolyte system has been chosen.

TABLE II

OPERATIONAL SYSTEMS

BALA = β-Alanine; EACA = ε-aminocaproic acid; HEC = hydroxyethylcellulose; OAc^- = acetate.

Electrolyte	*Parameter*	*System No.*		
		1	*2*	*3*
Leading	Solvent	H_2O	H_2O	H_2O
	Anion	Cl^-	Cl^-	Cl^-
	Concentration (m*M*)	10	10	10
	Counter ion	BALA	BALA	EACA
	pH_L	3.2	4.0	4.7
	Additive	HEC	HEC	HEC
	Concentration (%, w/v)	0.2	0.2	0.2
Terminating	Solvent		H_2O	
	Anion		OAc^-	
	Concentration (m*M*)		5	
	Counter ion		H^+	
	pH_T		*ca.* 4.0	

TABLE III

CALCULATED AND EXPERIMENTALLY DETERMINED VALUES OF PARAMETERS CHARACTERIZING THE INFLUENCE OF MATRIX CONSTITUENTS ON THE QUANTIFICATION OF BENZOATE

Succinate, glutarate and acetate (terminating anion) served as spacing constituents.

Calculated data

2D system No.	*Sequence of the leading electrolytes*	$_1A$	$_2A$	$_{1-2}A$	ΔS_x
I	3.2/4.0	32.95	31.55	13.77	0.582
II	3.2/4.7	32.95	34.24	1.68	0.949
III	4.7/3.2	34.24	32.95	1.68	0.951
IV	4.0/4.7	31.55	34.24	1.91	0.939

Experimental data[a]

2D system No.	*Sequence of the leading electrolytes*	$_1A^*$	$_2A^*$	$_{1-2}A^*$	ΔS_x^*
I	3.2/4.0	395	1071	169	0.572
II	3.2/4.7	395	6705	78	0.803
III	4.7/3.2	6705	395	160	0.976
IV	4.0/4.7	1070	6705	373	0.651

[a] $_1A^*$, $_2A^*$ and $_{1-2}A^*$ values are given in thousands of counts of the integrator; the tabulated data are average values obtained from three determinations carried out with separately prepared electrolyte solutions from the same chemicals and from the same water purification system (relative standard deviations were 2–7%).

From the tabulated ΔS_x values it can be seen that also the predictions of the clean-up efficiencies of the 2nd separation steps based on the calculations were in reasonable agreement with the values based on experimental data (1.7–36% relative standard deviations). On the other hand, the ratios of $_1A$ and $_2A$ values for given combinations of the leading electrolytes deviate considerably from the corresponding ratios based on experimental data ($_1A^*$ and $_2A^*$ in Table III). These deviations suggest that the constituents representing the sample matrix in the experimental examination of the proposed procedure did not meet the assumption concerning the uniform distribution of the matrix constituents within an ionic mobility–pK "space" (see Theoretical). The ITP profiles in Fig. 4 also support this suggestion. These results indicate that theoretical models considering more appropriate distribution functions[5] for the ionic mobilities and/or pK values could lead to better agreements between the calculated and experimental data. However, we feel that the choice of the distribution function(s) should be closely related to the nature of the sample matrix. In practical situations a small number of preliminary experiments can be helpful in finding the most appropriate distribution function(s).

Despite the above discrepancies, it is apparent that the proposed procedure based on a very simple model can be helpful in minimizing the number of experiments necessary to find optimum working conditions in 2D ITP trace analysis of constituents detectable by selective detectors with quantification by the spike method. Its

application to the solution of practical analytical problems and its further refinement are subjects of our current research.

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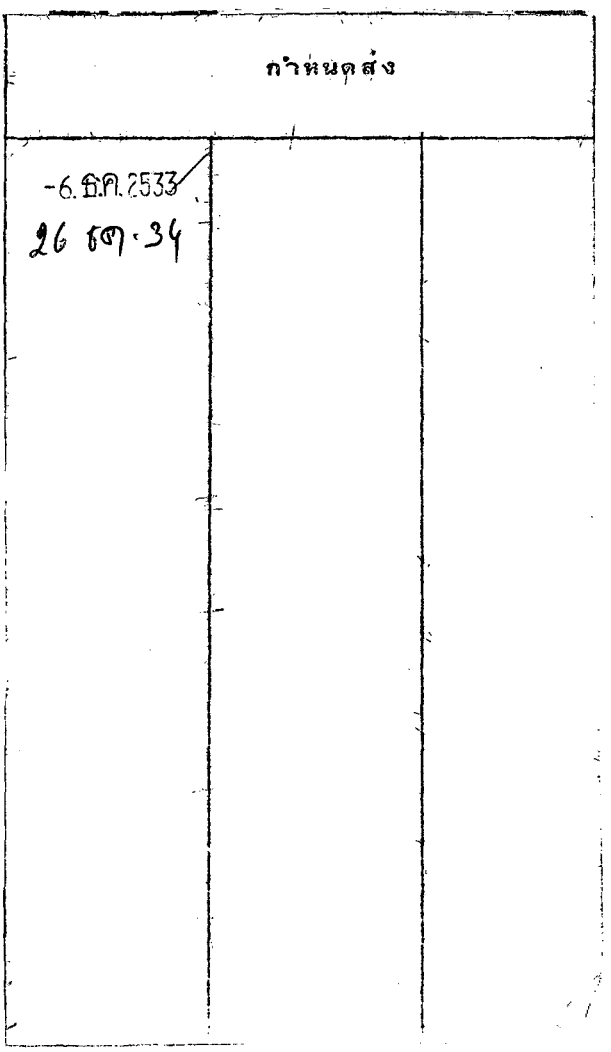
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