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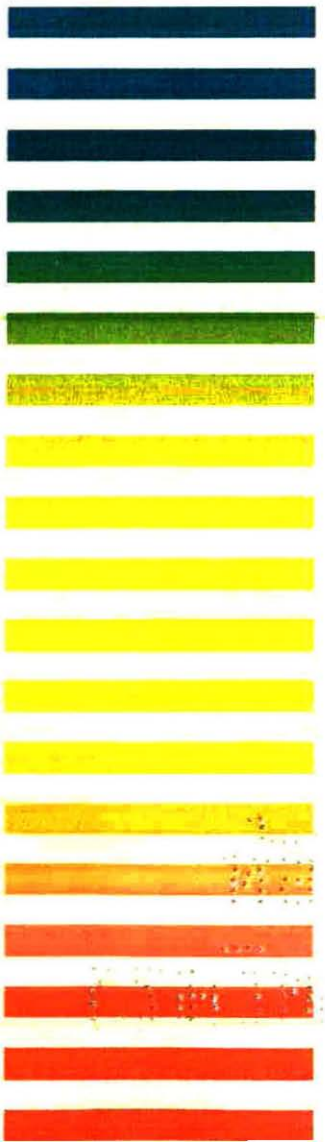

CHROMATOGRAPHIC REVIEWS (Vol. 30, No. 1)

edited by

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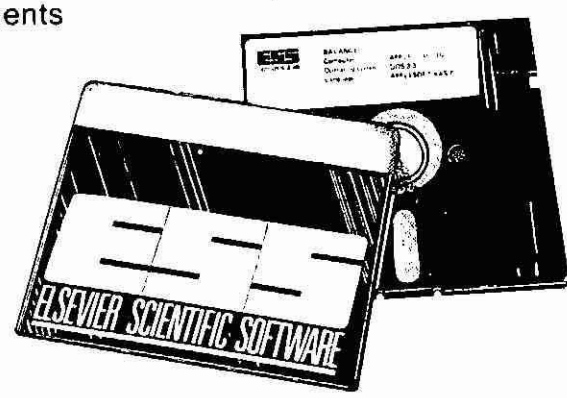

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CHREV. 189

SOVIET POLYSILOXANE STATIONARY PHASES FOR GAS CHROMATOGRAPHY

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(Received October 18th, 1984)

CONTENTS

1. INTRODUCTION

Polysiloxanes are currently the materials most commonly used as stationary phases in gas chromatography (GC). According to the data of Haken[1,2], extracted from literature sources, the proportion of polysiloxanes among all stationary phases increased from 40 to 60% between 1969 and 1977, and there has been a further increase since then[3].

In the past lists of preferred stationary phases have been published, and in one report four out of the six listed are polysiloxanes[4]. These materials ideally meet the major requirements imposed on stationary phases, as they are chemically inert and cover almost the entire range of chromatographic polarity when appropriate substituents are introduced.

In the Soviet Union, a wide range of polysiloxane gums[5,6] and fluids [7,8] has been produced. Many of them have been recommended for use as stationary phases in gas chromatography, although in recent years, in keeping with the worldwide trend

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TABLE 1

PHYSICAL CHARACTERISTICS OF POLYSILOXANE STATIONARY PHASES

Stationary phase	*Substituents*	$M_{liq}^{*} \cdot 10^{-3}$	*MWD*[6]		
			$\bar{M}_w \cdot 10^{-3}$	$\bar{M}_n \cdot 10^{-3}$	$\bar{M}_w/\bar{M}_n$
PMS-100	Methyl	3.5–4.6	8.0	4.4	1.84
PMS-1000	Methyl	6.4–10			
PMS-1 · 10^5	Methyl	34–38			
PMS-1 · 10^6	Methyl	220			
SKTN	Methyl	7.5–75			
SKT	Methyl	600–900	870	360	2.42
SKTV	Methyl, 0.04% vinyl	600–900	600	240	2.50
SKTE type A	Methyl, 8% ethyl	75–750			
VKZh-94	Ethyl	0.8	0.92	0.88	1.05
PES-V-2	Ethyl				
PES-S-1 (132-24)	Ethyl		2.0	1.5	1.33
PES-S-2 (132-25)	Ethyl				
PES-5	Ethyl	1.6–1.75			
SKTFV-803	4% Phenyl, 0.15% vinyl	150–400	460	200	2.30
FM 1322/300 (133-158)	Low phenyl content	1.6–2.4			
FM-6	Low phenyl content	3.0			
SKTTs	Methyl, phenyl				
Lestosil	Methyl, phenyl				
SKTA-1	See text	400–2000			
DFOK	See text	200–1000			
Copolymer-2	< 50% Phenyl	0.7–0.8			
Copolymer 2/300	< 50% Phenyl	1.2–1.6			
Copolymer-3	< 50% Phenyl	2.7–3.2			
PFMS-2	50% Phenyl	0.6–0.7			
PFMS-4	50% Phenyl (35% eff.)	1.2–1.5	2.1	1.4	1.50
PFMS-5 (133-165)	Phenyl (48% eff.)	1.1–1.3	1.7	1.3	1.29
SKTF-100	50% Phenyl	140–680	190	110	1.78
PFMC-6 (133-57)	Phenyl (58% eff.)	1.3–1.6	1.0	0.9	1.11
KhS-2-1	Chlorophenyl	1.5–2.5	3.1	2.0	1.55
KhS-2-IVV (162-170VV)	Chlorophenyl	2.8			
SF	See text				
FS-169 (161-56)	Trifluoropropyl (11.5% eff.)	1.6			
FS-169/300 (161-89)	Trifluoropropyl (11.5% eff.)	3.2	3.4	2.8	1.21

d_4^{20}	*Viscosity, cS, 20°*	n_D^{20}	*Cubic expansion coefficient,* $\cdot 10^3$ *deg*$^{-1}$ *at 100°C*	*Vapor pressure, mm Hg*		
				200°C	*250°C*	*300°C*
0.97	100 ± 10	1.405	0.96	2.2	7.9	22
0.98	1000 ± 100	1.406	0.95	0.3		1.0
0.98	$1 \cdot 10^5 \pm 1 \cdot 10^4$	1.406	0.95			
0.98	$1 \cdot 10^6$	1.406				
0.975	Gum	1.410				
0.975	Gum	1.410				
0.975	Gum	1.410				
0.975	Gum	1.415				
0.949	40–52	1.446				
	40–52	1.446		0.5	3.0	13.5
1 ± 0.05	210–315	1.446		17.4	36.3	66.1
1 ± 0.05	180–305	1.446		8.7	21.4	45.7
1 ± 0.02	200–500	1.448	0.73			
0.975	Gum	1.430				
1.05–1.06	60–90		0.90	15.6	21.5	27.9
<0.957	50	1.428		$1.7 \cdot 10^{-2}$	$7 \cdot 10^{-2}$	0.2
	Gum					
	Gum					
1.09	Gum	1.525				
1.04 ± 0.01	35	1.496		18.0	34.0	57.4
1.07 ± 0.01	160–220					
1.07 ± 0.01	220–400		0.82	4.7	13.7	33.1
1.02	20–30	1.497		1.0	10.6	46.3
1.10	600–1000	1.540	0.72	1.0	4.1	12.5
>1.12	1000	1.557	0.58	2.2	11.7	45.0
1.13	Gum	1.555				
1.15	45/150°C	1.585	0.61	$4.4 \cdot 10^{-1}$	2.5	10.0
1.03	40–47	1.430	0.84	$9.2 \cdot 10^{-2}$	$2.2 \cdot 10^{-1}$	4.5
1.03–1.04	70–85	1.442	0.90	$3.5 \cdot 10^{-3}$	$5.8 \cdot 10^{-2}$	$5.9 \cdot 10^{-1}$
1.085–1.107	45–60	1.391	0.94			
1.1	50–70	1.392–1.398		$2.7 \cdot 10^{-1}$	$9.2 \cdot 10^{-1}$	2.5

(Continued on p. 4)

TABLE 1 *(continued)*

Stationary phase	*Substituents*	M_{lit}* $\cdot 10^{-3}$	*MWD*[6]		
			$\bar{M}_w \cdot 10^{-3}$	$\bar{M}_n \cdot 10^{-3}$	$\bar{M}_w/\bar{M}_n$
SKTFT-25	12.5% trifluoro-propyl	95–475			
FS-328 (161-48)	Trifluoropropyl (15.5% eff.)	2–3			
SKTFT-50	25% trifluoro-propyl	11–58			
SKTFT-50X	25% trifluoro-propyl	115–575			
FS-16 (161-90)	Trifluoropropyl 1 (28% eff.)	1			
SKTFT-75	37.5% trifluoro-propyl	270–675			
SKTFT-100	50% trifluoro-propyl	300–800**	300–900**	200–370**	1.82–3.25
NFS-100	50% trifluoro-propyl	15–78			
FS-303 (161-126)	Trifluoropropyl (62% eff.)	2.4–2.7	2.9	2.3	1.27
NPS-25	12.5% cyanoethyl	1.5			
NPS-50	25% cyanoethyl	1.2	2.3	1.4	1.58
NPS-100	50% cyanoethyl	1.3			
NSKT-25	12.5% cyanoethyl	83–830			
NSKT-33	16.5% cyanoethyl	87–870			
NSKT-50	25% cyanoethyl	93–930			
NSKT-100	50% cyanoethyl	113–1130			
γ-NSKT-100	50% cyanopropyl	127–1270			

* The values of M_{lit} for fluids characterize $\bar{M}_n$ and were taken from refs. 7–10 and 19. The values of M_{lit} for gums characterize $\bar{M}_w$ and were calculated from the degree of polymerization (see refs. 23 and 37).

** The MWD data for SKTF-100 were taken from ref. 100.

to reduce the number of stationary phases, many Soviet chromatographers have used Western speciality polysiloxanes.

Soviet polysiloxanes used as stationary phases are reviewed here for several reasons. First, modern comprehensive reviews are not available and some conflicting and incomplete data have made it difficult to determine suitable alternatives. Soviet polysiloxanes have been briefly described in earlier Western reviews but are now presented here in greater detail. In this work the properties of frequently employed materials and some that are now virtually obsolete are included.

Second, to ensure that the wealth of data that have been published in the Soviet literature[1–3] could be used as fully as possible, the analogues produced in the West are indicated where available.

Third, as indicated in earlier reviews[1–3], some polysiloxane stationary phases developed in the Soviet Union have virtually unknown structures and are not available to chromatographers in other countries.

d_4^{20}	*Viscosity, cS, 20°*	n_D^{20}	*Cubic expansion coefficient, $\cdot 10^3 deg \cdot -1$ at 100°C*	*Vapor pressure, mm Hg*		
				200°C	*250°C*	*300°C*
1.065	Gum	1.400				
1.1	65–120	1.390–1.395	0.98	$2.6 \cdot 10^{-1}$	$8.2 \cdot 10^{-1}$	2.1
1.15	Gum	1.395				
1.15	Gum	1.395				
1.09	40	1.385	1.07	1.9	13.0	63.8
1.240	Gum	1.387				
1.329	Gum	1.383				
1.329	Gum	1.383				
1.330	1000–1500	1.3818	0.83	$6.6 \cdot 10^{-1}$	2.4	7.2
0.998	80	1.439				
1.05	350	1.439				
1.09	1340	1.4664				
	Gum					
	Gum					
	Gum					
	Gum					
	Gum					

Fourth, a new standardized (alphabetical) nomenclature has been recently adopted in the Soviet Union and is used together with the older numerical nomenclature. Both nomenclatures are given in Tables 1 and 2, with the older nomenclature in parentheses.

Fifth, another area of interest is the role of the solid support with regard to the properties of stationary phases. The wide use of exclusion chromatography as a means for determining the quality of stationary phases and the pattern of the changes they undergo in the course of analysis is also included.

The physical properties of Soviet polysiloxane stationary phases, critically selected from different sources, are listed in Table 1 and their chromatographic characteristics are given in Table 2. The latter table does not include obsolete materials.

In view of the steadily improving instrumentation and increasing sensitivity of detectors, the literature often contains stringent criteria for the evaluation of the quality of stationary phases. The upper temperature limits of the range in which stationary phases can be used are given in Table 2.

TABLE 2

CHROMATOGRAPHIC CHARACTERISTICS OF POLYSILOXANE STATIONARY PHASES

Stationary phase	*Substituents*	*Upper temperature limit*	*McReynolds (Rohrschneider) constants*						*Relative polarity, P (%)**	*Analogue*	*Ref.*
			X	*Y*	*Z*	*U*	*S*	*L*			
PMS-100	Methyl	250	15	55	43	65	42	44	9	DC-200	44
SKT	Methyl	300	17	57	46	67	45	47	6–8	SE-30	44
			18	57	45	65	45				47
SKTV	Methyl, 0.04% vinyl	300	16	56	44	66	43	46	6–8	SE-31	44
SKTE type A	Methyl, 8% ethyl	250							5		
VKZh-94	Ethyl	150	7	14	18	21	26	6			44
PES-S-1 (132–24)	Ethyl		10	14	17	22	24	7			44
SKTFV-803	4% Phenyl, 0.15% vinyl	300	29	73	62	97	68	65	10	SE-52, SE-54	44
SKTTs	Methyl, phenyl		79	114	114		141	130			46
Lestosil	Methyl, phenyl		73	124	122		189	140			46
PFMS-4	50% phenyl (35% eff.)	280	105	145	149	223	185	165	24	DC-710	44
			106	148	150	222	184				47
PFMS-5 (133–165)	48% eff. phenyl	250	142	176	182	267	233	210	30		44
SKTF-100	50% phenyl	330	145	181	186	272	240	217	36		23
			(1.57)	(1.96)	(2.05)	(3.13)	(2.17)				43
PFMS-6 (133–57)	See text; 58% eff. phenyl	300	164	192	198	288	262	236	33	Similar to OV-22	44
SKTA-1	See text	350	(0.82)	(1.29)	(1.16)	(1.32)	(1.68)		21		23
DFOK	See text; 27% eff. phenyl	400	86	130	134	181	154	143			44
			(1.18)	(1.64)	(1.69)	(1.56)	(1.90)				23
SAF	Siloxarophenanthrene	300	(0.81)	(1.51)	(1.94)	(2.73)	(2.38)		33		36

KhS-2-1	Chlorophenyl	300	22	67	66	87	65	56	9–10	Versilube F-50	44
SF	See text	320	27	128	173	188	239				30
FS-169 (161–56)	Trifluoropropyl	250	46	104	149	189	118	112	17		44
SKTFT-25	12.5% trifluoropropyl	300	(0.42)	(1.01)	(1.42)	(2.46)	(1.27)		15		23
FS-328 (161–48)	Trifluoropropyl (15.5% eff.)	250	55	116	169	215	137	130	20		44
SKTFT-50	25% trifluoropropyl	300	47	160	212	268	230				30
			73	141	207	272	172	159	21		44
			74	145	212		173	158			46
			73	142	207	266	172				47
			(0.82)	(1.49)	(2.47)	(3.50)	(2.00)				23
SKTFT-50X	25% trifluoropropyl	300	66	132	192	247	158	148			44
FS-16 (161–90)	Trifluoropropyl (28% eff.)	150	87	161	239	296	194	186	26		44
SKTFT-75	37.5% trifluoropropyl	300	(1.22)	(1.98)	(2.97)	(4.00)	(2.52)		26		23
SKTFT-100	50% trifluoropropyl	285	143	232	350	456	303	278	32	QF-1	44
			(1.59)	(2.46)	(3.47)	(4.50)	(3.00)				23
FS-303 (161–126)	Trifluoropropyl (62% eff.)	200	179	272	419	527	366	337	42		44
NSKT-25	12.5% cyanoethyl	250	122	261	237	345	241	211	37		44
			(1.05)	(2.44)	(1.57)	(2.11)	(1.98)				23
NSKT-33	16.5% cyanoethyl	250	135	275	251	363	259	225	42		44
NSKT-50	25% cyanoethyl	250	(1.91)	(3.52)	(3.29)	(5.00)	(3.12)		53	Similar to XE-60	23
NPC-50	25% cyanoethyl	200	164	320	287	426	302	268	37		44
NSKT-100	50% cyanoethyl	250	325	573	474	656	547	501	71		44
			(3.94)	(6.26)	(5.48)	(8.50)	(5.30)				23
γ-NSKT-100	50% cyanopropyl	250	276	461	405	584	473	421			44
NPS-100	50% cyanoethyl	180	297	502	451	644	512	448	62	Similar to XF-1150	44

* The values of P for siloxane gums were mostly taken from refs. 16 and 20 and those for fluids from ref. 14.

2. DEVELOPMENT OF SOVIET POLYSILOXANE STATIONARY PHASES

The first reports concerning the use of Soviet polysiloxanes as stationary phases were published by Berezkin and co-workers[11,12] and later by Turkeltaub *et al.*[13]. The first comparative study of the chromatographic characteristics of Soviet polysiloxanes was undertaken by Rudenko *et al.*[14], who investigated the properties of the methylsiloxane gums SKT and SKTV-1, methyl phenyl polysiloxane fluids and the copolymer PFMS-2 and their residues and have showed that the gum SKTV-1 is comparable to E-301. In subsequent work, Rudenko and co-workers[15,16] examined the chromatographic characteristics of eight gums containing methyl, phenyl, trifluoropropyl and nitrile substituents in comparison with the elastomers SE-30, SE-52 and XE-60. Also described for the first time were the trifluoropropyl derivatives SKTFV-803, SKTFT-50 and SKTFT-100.

In the preparation of polysiloxane stationary phases, it is necessary to remove low-molecular-weight impurities and catalyst residues, by reprecipitation in order to enhance their thermal stability. The reprecipitation was conducted by the dropwise addition of ethanol–water (85:15) to a 50% solution of the polymer in chloroform, ethyl acetate or acetone. The use of a smaller proportion of water resulted in stable emulsions. The precipitated polymer was washed twice with 85% ethanol and dried in a vacuum desiccator over P_2O_5 for seven days. The stationary phases were applied on to silanized Chromosorb P in an amount of 10%. Rudenko *et al.*[15,16] determined the upper temperature limits and the relative polarity (*P*) according to Rohrschneider[17] (for a blend of butanol-1, butanol-2, and *tert.*-butanol) and found that the Soviet siloxane gums are equivalent to their foreign analogues in terms of thermal stability and separating power.

Syavtsillo *et al.*[18] also studied comparatively, prior to the work of Rudenko *et al.*[15,16], twelve polysiloxane fluids. The study involved two diethyl polysiloxanes, three dimethyl polysiloxanes, five methyl phenyl polysiloxanes, including PFMS-2. PFMS-4 and PFMS-6, and the fluorosiloxane fluids FS-16 and the FS-169. Some physical properties of these phases were determined, and a correlation was established between their selectivity and dielectric constants.

Considerable work was carried out on polysiloxane stationary phases by Luskina and Turkeltaub[19], who examined the chromatographic properties of 16 polysiloxane fluids containing methyl, phenyl, trifluoropropyl and cyanoethyl substituents and determined their upper temperature limits, relative polarities (for benzene–cyclohexane), separation factors for C_{29}–C_{30} *n*-alkanes and the number of theoretical plates. All sorbents with polysiloxanes were first conditioned at 200–400°C in a flow of nitrogen for 200–500 h.

In the first Soviet review, Luskina and Turkeltaub[20] discussed the results of studies of the thermal stability of stationary phases for high-temperature gas chromatography, emphasizing the conditioning procedure and the methods for determining the upper temperature limit. It was indicated that the best way of determining this parameter is experimentation under conditions approximating the real operation of the chromatographic column, as opposed to thermogravimetric methods, which produce widely variant and even conflicting results. The factors responsible for chemical transformations of the stationary phase in the column (oxidation, decomposition, etc.) were discussed, together with procedures for eliminating them.

Extensive research involving the chromatographic characteristics of polysiloxane gums, including the development of new grades of elastomeric stationary phases, was carried out by Yudina, Sakodynskii and co-workers[21–31]. Some of their work[21–23] involved detailed studies of the properties of most grades of Soviet siloxane gums used as high-temperature stationary phases, such as the dimethyl polysiloxane SKT, the methyl phenyl polysiloxanes KSTF-100, SKTA-1 and DFOK, fluorosiloxanes of the SKTFT type and nitrilesiloxanes of the NSKT type. As a result of these experiments, the structures, basic physical properties, relative polarities, Rohrschneider constants[32] and upper temperature limits were specified for all these polymers[23]. The properties of the materials examined were compared with those of stationary phases manufactured by Ohio Valley and Supelco in the U.S.A.

Vigdergauz *et al.* proposed the use of and determined linear analogues of Rohrschneider constants for the fluids PMS-100[33] and FS-16[34].

Anvaer and Sakodynskii[35] published a comprehensive list of stationary phases, which covered the chromatographic properties of more than 150 commonly used phases, including many Soviet polysiloxanes. A special chapter in Haken's reviews[1,2], deals with these materials and Sakodynskii's review[36] lists the Rohrschneider constants and upper temperature limits of the new high-temperature polysiloxane phases containing carborane and aromatic rings. The properties of some polysiloxane gums[37] and fluids[38] can be found in the catalogues published by Reakhim, the agency responsible for sales of chemical reagents in the Soviet Union. All these data, plus information on the physical and chromatographic properties of polysiloxane stationary phases, included in earlier reviews[39–41] and reference books[42,43] are included in Tables 1 and 2. The *Gas Chromatography Handbook* written by Kocev and translated into Russian from Bulgarian was expanded by Berezkin and Urin, who edited the translation, to include information on the stationary phases produced in the Soviet Union[42].

Rohrschneider's system, expressing the capacity of stationary phases to be involved in various types of intermolecular interactions, has been evaluated[32] and was further elaborated by McReynolds[45] and has become universally adopted.

The McReynolds constants for 28 Soviet siloxane stationary phases of different structural types were determined for the first time by Itsikson *et al.*[44]. Particular attention was paid to the selection of the solid support. The criterion of a support's suitability was coincidence of the retention indices of a standard substance on a column with squalane and the McReynolds data[45]. The effect of the solid support on the retention indices in determining McReynolds constants is discussed later in this review.

The McReynolds constants for several phenyl- and fluoroalkyl substituted-siloxanes were determined by other workers[30,46,47]. Stolyarov and Kartseva[47] compared the polarities of various stationary phases, including SE-30, SKT, SKTFT-50, PFMS-4, Dexsil 400 GC and Silbor-1, using both the Rohrschneider–McReynolds system and a method based on the thermodynamic characteristics of solution[48]. Table 3 lists the differential molar free energies of solution ($-\Delta G$) calculated by the above workers in accordance with the latter method[48] for siloxane stationary phases. The McReynolds constants determined by Stolyarov and Kartseva[47] are included in Table 2. According to Stolyarov and Kartseva[47], the method for determining the polarity and selectivity of stationary phases, based on thermodynamic characteristics,

yields more information than the Rohrschneider–McReynolds system and permits a more reliable prediction of retention indices on various stationary phases.

As was shown in other work[49], the procedure commonly adopted for determining the content of polar substituents in polysiloxanes from the structure of the elementary link is applicable only to gums. With fluids, the actual content of the substituents is usually lower and depends on the molecular weight owing to the contribution of the terminal (usually methyl) groups.

Some polysiloxane stationary phases produced in the U.S.S.R. have been included in lists of materials recommended for use in gas chromatography. These lists have been compiled taking account of the relative polarities[50] and linear analogues of the Rohrschneider constants[34] and McReynolds constants[51,52]. Fridman and Vigdergauz[50,53] evaluated the sorptive capacity of the liquids PMS-100, PMS-500, PFMS-4, NPS-50 and NPS-100 with respect to hydrocarbons, esters, ketones and alcohols. It has been shown that stationary phases of medium polarity (relative polarity P = 30–60%), which are characterized by a high sorptive capacity with respect to any class of substance examined, are the most suitable for preparative chromatography.

Malakhov *et al.*[54] examined the suitability of the siloxanes PFMS-4, PMS-100, SKTFT-50 and SKTFV-803 for gas chromatography–mass spectrometry. Kanchenko *et al.*[55] reported values of the coefficients of cubic expansion of some polysiloxane fluids, necessary for determining the volume of stationary phase in the column at elevated temperatures. In view of the importance of this coefficient, we have included in Table 1 values taken from different sources[7–10]. Studies of the thermal stability of polysiloxanes by thermal analysis have been reported in a number of papers. Thermogravimetry, differential thermogravimetry and differential thermal analysis have been used to determine the upper temperature limits of 36 stationary phases, including FS-16 and PFMS-4[56]. The values obtained (120 and 250°C) are about 30°C below the commonly assessed values, probably because the materials had not been conditioned in advance. Further, as has been mentioned[18], these methods often yield results that are at variance with chromatographic practice.

Luk'yanova *et al.*[57] and Rudenko *et al.*[15,16] demonstrated, in thermogravimetric experiments, that the removal of catalyst residues from methyl phenyl polysiloxane fluids slows their decomposition at temperatures ranging from 350 to

TABLE 3

DETERMINATION OF THE POLARITY OF POLYSILOXANE STATIONARY PHASES FROM THE VALUE OF $-\Delta G$

Stationary phase	$-\Delta G_{CH_2}$ *(cal/mole)*	$-\Delta G_i$ *(cal/mole)*				
		Benzene	*Butanol-1*	*Pentanone-2*	*Nitropropane*	*Pyridine*
SE-30	452	2526	2432	2544	2757	2856
SKT	466	2482	2370	2487	2697	2822
SKTFT-50	439	2537	2563	3011	3380	3174
PFMS-4	492	2736	2632	2825	3303	3347
Dexsil-400	498	2743	2673	2888	3241	3266
Silbor-1	477	2704	4952	2918	3390	3583

400°C[55]. A study of the chlorophenylsiloxane KhS-2-1 was described by Voznesenskaya *et al.*[58].

The thermal decomposition of polysiloxanes in an inert gas or vacuum has been studied by different workers[59–66]. Volchinskaya *et al.*[59] compared the thermal stabilities of methyl- and ethylsiloxane fluids in argon. Other groups have studied the thermal decomposition of dimethyl polysiloxanes[60,61], methyl phenyl polysiloxanes[62,63], methyl trifluoropropyl polysiloxanes[64], dimethyl (methyl dichlorophenyl) polysiloxanes[65] and phenyl metal polysiloxanes containing Mn, Co, Ni, Cu and Zn, in comparison with pure phenyl polysiloxane[66]. The processes of thermal and thermo-oxidative decomposition of polysiloxanes were detailed by Kharitonov and Ostrovsky[67].

The common purpose of all of the above work was to define the areas of application of the materials under consideration. However, the results are also valuable in that they provide experimentally substantiated upper temperature limits, which are usually determined empirically. A knowledge of the thermal oxidative pattern of polysiloxanes permits the prediction of the service life of a chromatographic column as a function of its temperature and the oxygen content of the carrier gas. Some of the results reported in the above work have been used in analysing the properties of individual groups of polysiloxane stationary phases.

Itsikson and Moskaleva[68] used size exclusion chromatography to determine the molecular weight distribution (MWD) of about 30 siloxane stationary phases produced in the Soviet Union and elsewhere. Many materials were found to contain low-molecular-weight impurities, particularly the initial cyclic tetramers. Ainshtein *et al.*[69] described a GC technique for determining cyclic trimers and tetramers in methyl phenyl polysiloxane rubbers. Itsikson *et al.*[70] found that, during conditioning of the stationary phase in the column and also during its use near its temperature limits, the molecular weight of polysiloxane fluids increased with slight changes in their MWD and polarity. Andrianov *et al.*[71] demonstrated the applicability of universal calibration according to Benoit *et al.*[72] for the determination of the MWD of dimethyl polysiloxanes and methyl phenyl polysiloxanes by size exclusion chromatography.

A number of methods have been developed for determining various substituents in the polysiloxane chain by reaction or pyrolysis gas chromatography. These methods have been used to determine vinyl groups[73–75], alkoxy groups[76], methyl groups of the $(CH_3)_3$–$SiO_{0.5}$–, $(CH_3)_2$–SiO– and CH_3–$SiO_{0.5}$– types[77], ethyl and phenyl groups[78], ethyl methyl, trifluoropropyl and diphenyloxy groups[79] and alkyl and phenyl group combinations[80,81]. There are also reports on the analysis of the structural link ratio in methyl phenyl polysiloxanes[82], the content of trifunctional links in dimethyl polysiloxanes[83] and the basic principles of the functional analysis of organosilicon compounds[84]. The last subject has been reviewed by Luskina and Terent'yeva[85,86].

We have omitted work dealing exclusively with analytical applications using Soviet-produced siloxane stationary phases, as their application in the analysis of organosilicon compounds was treated in a definitive report by Shatz *et al.*[87] and a similar application in the analysis of terpenes was reported by Bardyshev *et al.*[88]. Much information about the use of Soviet siloxanes for the analysis of unstable and reactive compounds can be found in a book by Ivanova and Frangulyan[89]. The areas

of application of polysiloxane stationary phases are well known to specialists, and they can be clearly defined in terms of the phase structure and McReynolds or Rohrschneider constants. The examples given in this review are taken primarily from the first publications on a particular phase. The following sections deal with individual types of polysiloxane stationary phases.

3. ALKYL-SUBSTITUTED POLYSILOXANES

This group of stationary phases include siloxanes with methyl, vinyl and ethyl substituents. The most widely used dimethyl polysiloxane stationary phases in the Soviet Union include the gum SKT and the fluid PMS-100. In its structure and chromatographic characteristics, SKT is similar to the well known stationary phase SE-30. SKTN is a low-molecular-weight product with the same structure that has seldom been used as a stationary phase.

According to Rudenko *et al.*[14], SKT gum has an inferior thermal stability to SKTV-1. We believe that their conclusion was the result of using a poor quality sample: comparison of the MWDs of the gums SKT, SKTV and SE-30[68] and experience with using this material suggest that all these gums are equivalent in terms of thermal stability and, after reprecipitation, SKT is virtually interchangeable with the specialty gum SE-30 GC.

The dimethyl vinyl polysiloxane gums SKTV (in ref. 16, this was misprinted as SKSV) and SKTV-i contain 0.4 and 0.18% of vinyl group, respectively, and are close to the General Electric Company gum SE-31. The chromatographic characteristics of these materials are similar to those of the gums free of vinyl groups[1], which are frequently used in gas chromatography.

The PMS fluids are essentially dimethyl polysiloxanes with a linear structure, similar to DC-200, produced by Dow Corning (U.S.A.). The numerals following the name of the fluid indicate its viscosity, in centistokes, at 20°C. The PMS fluid range includes more than 40 grades with viscosities varying from 1 to $1.2 \cdot 10^6$ cS. Their properties have been described in detail elsewhere[7]. The fluid PMS-100 was first proposed for use in gas chromatography by Syavtsillo *et al.*[18]. Its upper temperature limit is 250°C but, after proper conditioning, this and many other polysiloxane fluids can be used at higher temperatures. For example, Turkeltaub and Luskina[90] analysed high-boiling organosilicon compounds on PMS-100 at temperatures of up to 340°C. The physical properties of some PMS fluids covering a broad range of molecular weights are listed in Table 2.

Fig. 1 shows chromatograms of some dimethyl polysiloxanes, obtained by an exclusion technique[68] on a Waters 200 instrument. These indicate that the MWD of SKTV gum is close to that of SE-30, but the former contains more high-molecular-weight fractions. In terms of MWD, SKT gum occupies an intermediate position between SE-30 and the higher molecular weight gum OV-1. The polydispersities of OV-1, SKT and SKTV are almost the same (2 and 2.5), whereas that of SE-30 is slightly lower (1.9). The PMS fluids exhibit a much lower molecular weight and a higher polydispersity (1.84) than OV-101 (1.35).

Polysiloxanes containing longer chain aliphatic substituents include the gum SKTE, grade A, containing 8% of diethyl siloxane links and 0.06% of vinyl groups, and the diethyl polysiloxane fluids BKZh-94 and PES-1-S. The low content of ethyl

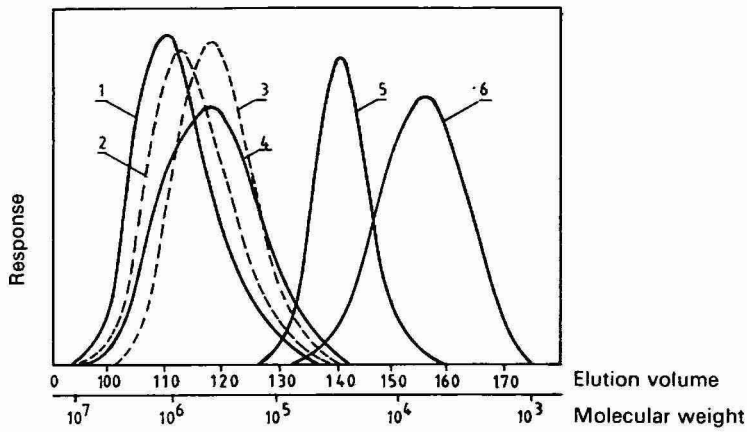


Fig. 1. Size exclusion chromatograms of dimethyl polysiloxane stationary phases. 1 = OV-1; 2 = SKT; 3 = SE-30; 4 = SKTV-1; 5 = OV-101; 6 = PMS-100.

groups in SKTE gum (8%) does not significantly affect its polarity, and its use as a stationary phase offers virtually no advantages over SKT and SKTV. Complete replacement of methyl by ethyl groups in the polysiloxane molecule substantially reduces the polarity of the stationary phase.

Determination of the McReynolds constants[44] has shown that the diethyl siloxanes BKZh-94 and PES-S-1 have almost the same polarity as the reference hydrocarbon ($C_{87}H_{176}$) developed by Kováts and hydrogenated Apiezon M (MH)[91] and are less polar than dimethyl siloxanes and Apiezon L. The sums of the first five McReynolds constants for diethyl siloxane stationary phases, hydrocarbon $C_{87}H_{176}$, Apiezon MH, SE-30 and Apiezon L are 86–87, 71, 82, 217 and 143, respectively. The fluid BKZh-94 was first used by Turkeltaub *et al.*[92] to separate a mixture of $SiCl_4$, $COCl_2$, HCl and Cl_2 and was widely employed in early work. The main drawback of this popular fluid is its low upper temperature limit of 140–150°C.

The maximum working temperature of a stationary phase is determined primarily by two parameters: thermal stability and vapour pressure at the limiting temperature.

Volchinskaya *et al.*[59] showed that the thermal stability of diethyl polysiloxanes is higher than that of dimethyl and dimethyl phenyl polysiloxanes. The decrease in viscosity at 100°C after exposure for 100 h in argon at 350°C was 12% for PMS-100, 17% for PFMS and only 5% for PES. When the temperature was increased to 375 and 400°C under the same conditions, the viscosities of the two ethyl-substituted polysiloxanes decreased by 18 and 20%, respectively.

The vapour pressure of polysiloxane fluids of the same structure decreases inversely with the molecular weight and directly with the content of low-volatility oligomers. Therefore, it would be appropriate at this juncture to consider the properties of some diethyl polysiloxane fluids produced in the U.S.S.R.[6] and not used previously in gas chromatography. The highest molecular weights are exhibited by PES-5 and PES-S-1 and the lowest vapour pressure (10^{-6}–10^{-8} mmHg at 20°C) by PES-V-2, which is an improved version of the fluid BKZh-94, free of low-molecular-weight oligomers. Comparison of its physical properties with those of the dimeth-

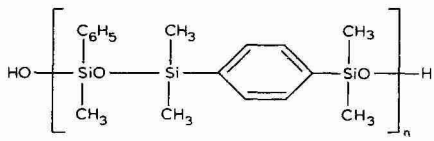
yl polysiloxane PMS-100 (upper temperature limit 250°C) indicates that the upper temperature limit of these fluids, after conditioning, will be at least 220–250°C. Hence these materials may be regarded as promising for use as "zero"-polarity stationary phases.

4. PHENYL-SUBSTITUTED POLYSILOXANES

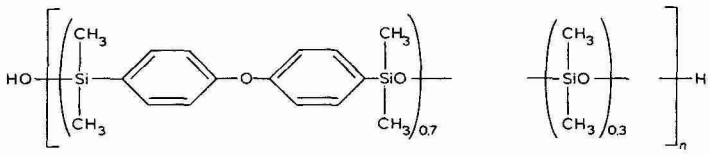
This group of stationary phases is available in the largest number of grades. Soviet industry produces a broad range of methyl phenyl polysiloxane fluids with linear, branched and cyclic structures[9] and various grades of gums containing phenyl groups.

The gum SKTFV-803, first studied by Rudenko *et al.*[14] and subsequently frequently used in chromatography, contains 4% phenyl substitution and 0.15% vinyl substitution, and is thus similar in composition to the gum SE-54. The gum SKTF-100 (50% phenyl substitution) is similar in structure to OV-17 but has a much higher molecular weight.

It has been reported that the introduction of phenylene groups into the main chain increases the thermal stability of polysiloxanes by 60–100°C[41]. Of particular interest are the stationary phases proposed for chromatography by Yudina *et al.*[26], namely the gums SKTA and DFOK, which contain propylene or diphenyl oxide groups in the main chain. The liquid phase SKTA-1 has the general formula

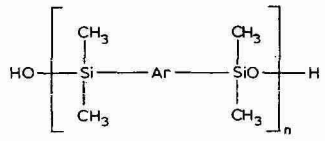


where n = 1000–5000 and DFOK has the formula



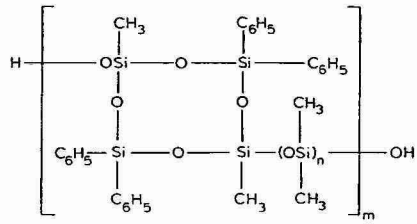
where n = 1000–5000.

The first reports on the use of DFOK were published by Yuzhelevsky *et al.*[93] (in the separation of stereoisomers of methylphenyl dimethyl cyclosiloxanes) and Yudina and Voronkina[94] (in the analysis of a repellent mixture). The application of SKTA gum in high-temperature preparative chromatography has been described[24]. As regards polarity, SKTA-1 and DFOK occupy an intermediate position between OV-7 and OV-11 and are characterized by good selectivity and high upper temperature limits (350–400°C). Similar materials having the general formula



where Ar = C_6H_4 or $C_6H_4OC_6H_4$, were recommended by Sivtsova *et al.*[95], but they have not yet found much use.

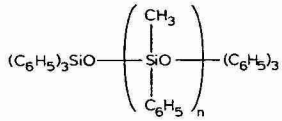
Also proposed as high-temperature stationary phases are polysiloxanes with siloxarophenanthrene groups in the side-chain[27], named SAF[36], and cyclolinear siloxanes[28] of the general formula



where n = 3–20 and m = 10–100.

Prokopenko *et al.*[46] determined McReynolds constants for the phenyl-substituted gums SKTTs and Lestosil, but no data on their structure or thermal stability are available.

The chromatographic characteristics of methyl phenyl polysiloxane fluids have been studied by many workers[20,44,50]. The most commonly used is the fluid PFMS-4[11,12], whose analogue is DC-710 and PFMS-6 with triphenyl terminal groups and the general formula



The fluid PFMS-4 was introduced into chromatography by Berezkin and co-workers[11,12] and Turkeltaub *et al.*[13]. PFMS-6 and some other methyl phenyl polysiloxane fluids have been used by Turkeltaub *et al.*[96] for separating phenylchlorosilanes.

In earlier work, Itsikson and Snegirev[49] proposed the determination of the effective content of polar groups in phenyl- and trifluoropropyl-substituted polysiloxanes, thus permitting an objective comparison of the chromatographic polarities of various materials.

Table 4 lists comparative contents of phenyl substituents in some methyl phenyl polysiloxanes, the comparison being in terms of the elementary link structure and based on the method described earlier[49]. It is evident that the effective contents of the phenyl substituents in the polysiloxanes OV-11, PFMS-4 and DC-710 are virtually the same. The McReynolds constants for these stationary phases also coincide.

Fig. 2 shows size exclusion chromatograms of four stationary phases with similar structures, containing 50% phenyl substituents in the elementary link. Comparison of the curves indicates that three of the materials (not SP-2250) contain a low-molecular-weight impurity identified as a cyclic tetramer[49,68]. The molecular weight of the major component of PFMS-4 is lower than that of DC-710, but the

TABLE 4

CONTENT OF PHENYL SUBSTITUENTS IN METHYL PHENYL POLYSILOXANE STATIONARY PHASES

Phenyl substituent content	*Stationary phase*							
	OV-11	*PFMS-4*	*DC-710*	*OV-17*	*PFMS-5*	*OV-22*	*PFMS-6*	*OV-25*
As determined from the elementary link structure	35	50	50	50	–	65	68*	75
As determined from NMR data		39		42			66	63
Effective content according to ref. 49	33.5	35	36	40	48	55	58	64

* Calculated from the general formula and molecular weight.

latter contains much more of the tetramer. The molecular weights of these stationary phases determined by vapour phase osmometry are virtually identical (1500–1600). The effective content of phenyl groups in PFMS-6 is 58% (this is less than the true content as a result of the steric hindrances that occur during the interaction of the sorbates with the triphenylsilyl terminal groups[49]. PFMS-6 is very similar to OV-22 in terms of polarity, although its chemical structure is entirely different. The fluid PFMS-5, whose effective content of phenyl substituents is 48%, occupies an intermediate position between OV-17 and OV-22.

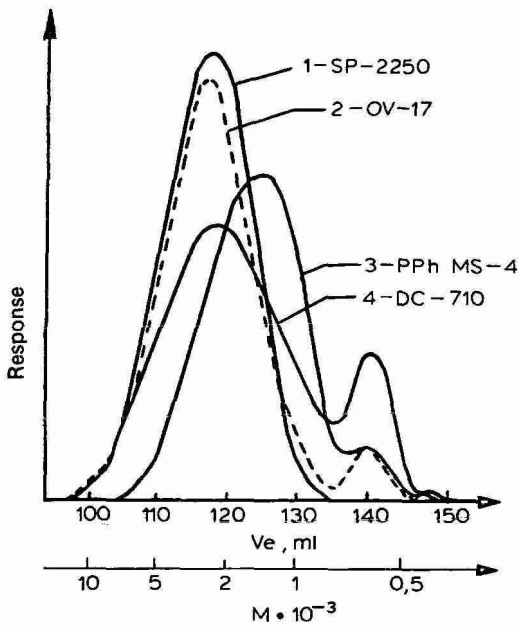


Fig. 2. Chromatograms of methyl phenyl polysiloxane fluids of similar structure. 1 = SP-2250; 2 = OV-17; 3 = PFMS-4; 4 = DC-710.

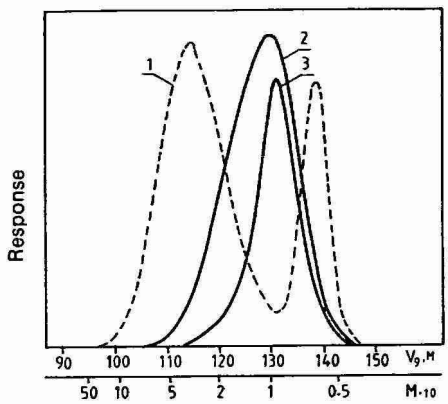


Fig. 3. Chromatograms of methyl phenyl polysiloxane stationary phases with a high content of phenyl groups. 1 = OV-25; 2 = PFMS-5; 3 = PFMS-6.

Fig. 3 shows for comparison size exclusion chromatograms of fluids with high phenyl substituent contents, *viz.*, PFMS-5, PFMS-6 and OV-25. The MWDs of the first two stationary phases are as expected, whereas OV-25 contains a considerable amount of low-molecular-weight substances, probably the starting cyclic compounds. The molecular weight of OV-25, determined by vapour phase osmometry, is 2100, that is, it approaches the value calculated from the chromatogram (1700 after calibration using polystyrene standards) but is much lower than the reported value[1] (10000). An investigation of two different OV-25 samples yielded similar results.

The upper temperature limit of commercial polysiloxane fluids is lower, in general, than that of gums, but after thorough conditioning they are capable of withstanding high temperatures[18]. For instance, the PFMS-6 fluids have been used for separating oligomers in methyl phenyl polysiloxanes at 320–400°C[97].

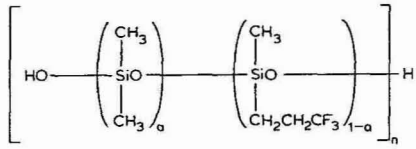
5. HALOGEN-SUBSTITUTED POLYSILOXANES

This group of stationary phases includes chlorophenyl- and trifluoropropyl-substituted polysiloxanes.

Chlorophenyl-substituted siloxane stationary phases have often been used in gas chromatography in recent years. Of the chlorophenyl siloxanes produced in the USSR[10], the most suitable for use as stationary phases are the fluid KhS-2-1 and a similar fluid KhS-2-1VV that contains a smaller amount of lower molecular weight oligomers. The siloxane KhS-1 described in an earlier paper[98] is a modification of KhS-2-1.

The McReynolds constants for KhS-2-1 are higher than those for Versilube F-50 but lower than those for DC-560 and SP-400[44]. As shown by Haken[1,2], chlorophenyl-substituted fluids of the DC-560 type have very similar polarities to the gum SE-52, which contains 5% phenyl substituents. The fluid KhS-2-1 is almost identical with another material containing the same proportion of phenyl substituents, DC-510, and their McReynolds constants are an average of 7–8 units below those of SE-52. However, the vapor pressure of KhS-2-1 is much lower, and its upper temperature limit is about 300°C.

The range of fluorine-containing siloxane stationary phases produced in the Soviet Union is extremely wide. The chromatographic properties of fluorine-containing gums have been studied in detail by Yudina and co-workers[21–23,30,99] and Rudenko *et al.*[16] and those of fluorine-containing fluids have been investigated by Luskina and Turkeltaub[19] and Itsikson and co-workers[44,49]. Gums of the SKTFT type are linear polymers with the general formula

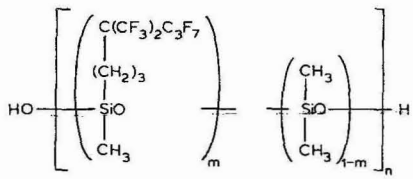


The numeral following the grade of the gum indicates the percentage of methyl trifluoropropyl siloxane groups. In high-molecular-weight polymers n = 1000–5000 and in low-molecular-weight polymers (SKTFT-50 and NFS-100) it is one order of magnitude lower. The gum SKTNFT-50 studied in early work[16,44,49] seems to have an even lower molecular weight.

The fluorosiloxane gum SKTFT-100 is similar in polarity to the established materials QF-1 and QV-210 but, according to one report[30], it has a slightly higher upper temperature limit (285°C). The determination of the MWDs of different samples of this gum by turbidimetric titration has been described[100] and the results are summarized in Table 1. The other SKTFT gums with a lower content of trifluoropropyl substituents are even more thermally stable and have no analogues in the West[23]. The upper temperature limit of these stationary phases is 300°C[30]. A study in air at 250–430°C of the thermal stability of SKTFT gums containing 5, 33 and 100% of methyl trifluoropropyl siloxane links showed that substitution of trifluoropropyl groups for methyl ones at 290°C and above decreases the thermal stability of the polymer[64].

The most popular fluorosiloxane gum in the USSR is SKTFT-50, which was first used by Yudina *et al.*[101] to separate cyclosiloxanes. This material gives good results in capillary columns with an efficiency of 1500 theoretical plates per metre with respect to chrysene) and in the preparative chromatography of high-boiling compounds[24,103]. The separation of poorly chromatographed substances, such as alkyl- and oxygen-containing compounds of germanium, silicon and tin[104] and niobium and tantalum chlorides[105], has also been reported.

In 1980, Yudina *et al.*[29] proposed a high-temperature fluorine-containing stationary phase, SF, of general formula



where m = 0.33–1 and n = 20–3000. The properties of SF have been described elsewhere[30]. Its upper temperature limit is 320°C. The McReynolds constants for SKTFT-50 and SF have been determined on columns containing 5% of stationary

phase on a non-silanized support[30]. The constants determined for SKTFT-50 differ from those reported by other workers[44,46,47], which were similar to each other. The discrepancies are probably due to the effect of the type of support and the low content of the stationary phase on the solid support.

The physical properties of the fluorosiloxane fluids produced in the Soviet Union have been described elsewhere[10]. In earlier work, primarily FS-16 and FS-169 were used in preference to other stationary phases of this type. They were first proposed for gas chromatography by Syavtsillo *et al.*[18].

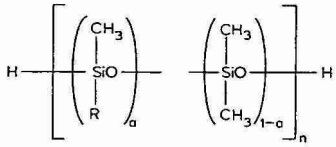
Studies of the chromatographic characteristics of some fluorosiloxane fluids have shown that most of them have similar polarities and, in some instances, thermal stabilities[21,44] and have no analogues. As the chemical structures of these materials are not known, it is most appropriate to compare them in terms of the effective content of trifluoropropyl groups[49], shown in Table 1.

The fluid FS-16 (28% effective $CH_2CH_2CF_3$ groups) has a very similar polarity to the gum SKTFT-50, but exhibits a much lower upper temperature limit, and its use at present is restricted. Interesting properties are displayed by the fluid FS-303, proposed for use by Ainshtein *et al.*[106]. The effective content of trifluoropropyl substituents in FS-303 is much higher than that in QF-1 (62 and 50%, respectively), and the McReynolds constant differences $Z-X$ and $Z-Y$ exceed those of QF-1 and OV-210 by 25–30 units. In some instances this may be important, particularly in the separation of mixtures containing alcohols or carbonyl compounds. The upper temperature limit of this stationary phase is 200°C.

As regards other fluorosiloxane fluids, the most commonly used is FS-169, containing 11.5% effective trifluoropropyl substituents and exhibiting a sufficiently high thermal stability. A similar fluid, FS-169/300, does not contain lower molecular weight oligomers.

6. CYANOALKYL-SUBSTITUTED POLYSILOXANES

The NSKT-type gums produced in the Soviet Union have the general formula

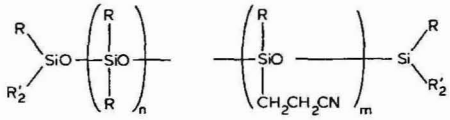


where n = 1000–10000 and R = β-cyanoethyl (β-NSKT) or γ-cyanopropyl (γ-NSKT). In the former instance, the symbol of the substituent type ("β") is often omitted. The numeral following the grade represents the percentage of the nitrile-containing group in the polymer molecule. NSKT gums were first proposed for use by Yudina *et al.*[25], who used them later as stationary phases[107]. The gum NSKT-50 contains 25% of cyanoethyl groups and is similar to the widely used material XE-60. The other gums have no analogues.

The fluids NPS-25, NPS-50 and NPS-100 have the same general formula as the NSKT gums but differ from them by having trimethyl silyl terminal groups and a lower molecular weight. The chromatographic characteristics of these fluids were first described by Luskina and Turkeltaub[20], and those of the gums by other work-

ers[16,21,23]. The McReynolds constants for some cyanoalkyl substituted siloxanes were determined in early work[44] and the MWD was studied later[68].

In terms of structure, the NPS fluids are similar to those of the XF type produced by General Electric (U.S.A.). However, their McReynolds constants (especially those of NPS-50) are lower than those of the XF type, probably owing to their lower molecular weights. Also proposed for use as stationary phases are fluids of the NPS type containing aryl substituents in the terminal groups[108]. Their general formula is



where R is C_1–C_{10} alkyl, R′ is aryl, n = 0–15 and m = 5–30.

The thermal stability of the above cyanoalkyl-substituted polysiloxanes is inferior to that of other types of siloxane stationary phases and does not exceed 230–250°C. Of these materials, those most commonly used in chromatography are the fluids NPS-50 and NPS-100 and the gum NSKT-50.

7. CARBORANE–SILOXANE STATIONARY PHASES

Carborane–siloxanes are an important class of stationary phases, their characteristic feature being high thermal stability.

In the early 1970s, linear *m*-carborane polysiloxanes[109] of the following type were first proposed as stationary phases in gas chromatography:

$$H[Si(CH_3)_2CB_{10}H_{10}C][R(R_1)SiO_m]_nH$$

where R is C_1–C_8 alkyl, R_1 is C_6–C_{10} aryl, m = 1–5 and $n \leqslant 50$. The commercially available compounds of this type, Dexsil-300, -400, and -410, have been widely and successfully used in chromatographic practice[110]. Carborane–siloxane stationary phases have also been used in the Soviet Union.

Yudina *et al.*[31] developed a carborane–siloxane stationary phase for the GC separation of organosilicon and organic compounds. This phase is essentially a high-molecular-weight siloxane polymer with carborane groups in the main chain and has the general formula

$$H[Si(CH_3)_2CB_{10}H_{10}C][Si(CH_3)_2O_m]_nH$$

where m = 1 (KBS-1), 2 (KBS-2) or 3 (KBS-3), n = 1000 and the molecular weight is 10^5–10^6. When m = 2 the stationary phase has the form of white crystals and when m = 3 it is a colourless rubber-like polymer. The upper temperature limit exceeds 500°C. It is recommended that KBS-2 and KBS-3 are coated on to a solid support in an amount of 20% (w/w) of the support (Chromaton N-AW). Yudina *et al.*[31] gave examples illustrating the use of this phase for the chromatographic determination of methyl(propyl) dimethyl cyclosiloxanes, C_{12}–C_{24} aromatic amines, polyphenyl esters, hexaphenyl cyclotrisiloxane and traces substances in aniline. When

TABLE 5[110]

RETENTION CHARACTERISTICS OF METHYL ESTERS OF AROMATIC ACIDS ON DIFFERENT STATIONARY PHASES

Chromatographed compound	*Phenetidyl terephthalate*		*OV-17*		*XE-60*		*KBSN-3F*		*Polybutylene glycol succinate*		*PEG-40M*	
	°C	*I*	°C	*I*	°C	*I*	°C	*I*	°C	*I*	°C	*I*
Methyl *n*-toluate			150	380	160	331	160	408	160	339	160	360
Methyl *m*-toluate			150	367	–	–	–	–	160	328	160	349
Dimethyl terephthalate	205	966	200	724	200	731	160	677	200	842	200	813
Dimethyl isophthalate	205	888	200	745	200	761	160	697	200	871	200	836
Methyl 2-naphthoate	265	1148	205	912	200	915	200	883	200	1044	200	1034
Trimethyl trimellate	205	1318	205	1121	200	1271	200	1034	210	1450	220	1380
Dimethyl 2,6-naphthalene dicarboxylate	205	1876	205	1377	200	1440	220	1272	210	1708	220	1654

used for the determination of trace impurities in aniline, KBS-2 gave better results than Dexsil-type stationary phases.

Later, Yudina *et al.*[111] proposed another type of carborane–siloxane stationary phase whose composition is given by the formulae

$$HO[Si(CH_3)_2CB_{10}H_{10}C\text{–}Si(CH_3)_2\text{–}O\text{–}Si(CH_3)C_6H_5\text{–}O]_nH$$

and

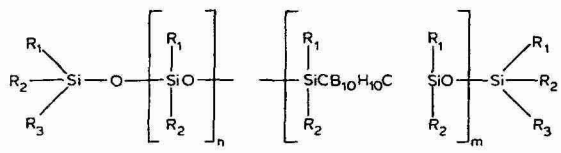
$$HO[Si(CH_3)_2CB_{10}H_{10}C\text{–}Ci(CH_3)_2\text{–}O\text{–}Si(CH_3)C_6H_5Si(CH_3)C_6H_5\text{–}O]_nH$$

where $n = 10^3$ and the molecular weight is 10^5–10^6. The first of the above two phases is usually referred to as KBS-2F and the other as KBS-3F. The upper temperature limit is above 450°C. The examples given[111] was the separation of mixtures of isopropyldiphenyl isomers.

Chernyshev and Vigdergauz[112] studied the pattern of the chromatographic retentions of methyl esters of aromatic acids on various stationary phases, including silicones of the carborane type and their results are presented in Table 5. It can be seen that carborane–siloxanes of the KBS type are of low polarity. The Soviet carborane–polysiloxane phases of the KBS type for gas chromatography differ from the Dexsil-type phases in their much higher molecular weights. This is why the characteristics of these phases are more significantly affected by the main rather than the terminal groups of carborane-polysiloxanes. The Rohrschneider constants for some carborane–siloxane phases are listed in Table 6[34,107–110].

According to Anvaer and Sakodynskii[35], the Soviet high-temperature stationary phases KBS-1 and KBS-2 have similar properties to the Dexsil type. The properties of these phases are also discussed elsewhere[36,109–113].

To separate high-boiling organic compounds, Luskina *et al.*[114] also proposed a carborane–siloxane stationary phase with the following structure:



where R_1, R_2 and R_3 are alkyl-substituted or -unsubstituted aryls, n = 3–24 and m = 1–8. As shown by Luskina *et al.*[115], materials of this type, containing terminal trimethylsiloxy, methyldiphenylsiloxy and γ-trifluoropropylsiloxy groups, can be used as stationary phases in a wide temperature range of from −30 to 400°C and, for a short period of time, up to 450–500°C. The use of such carborane–siloxane stationary phases for the separation of high-boiling organosilicon compounds has been described[116,117].

Recently, a new carborane–siloxane stationary phase, Silbor-1, was reported[118]. This is a viscous, rubber-like fluid readily soluble in toluene, benzene, methylene chloride and acetone and almost insensitive to oxygen. The working temperature range is 20–250°C, but special heat treatment has made it possible to expand this range to up to 350°C. The chromatographic characteristics of Silbor-1 in comparison with some other stationary phases are listed in Tables 7 and 8[1,45,118]. It is evident from these tables that the most salient feature of Silbor-1 is its extremely high selectivity towards compounds containing a hydroxy group.

Comparison of all McReynolds constants, except *Y*, indicates that Silbor-1 is similar to Dexsil-410 in terms of polarity. However, as regards the relationship between *Y* and the other constants, this stationary phase should be classified as having extremely high selectivity[52].

Silbor-1 has been used successfully to separate various perfume oils[119] and to determine aldehydes in higher fatty alcohols and acids[120]. Some of its characteristics have been reported by Loktev and Eliner[121].

TABLE 6

CHARACTERISTICS OF SOME SOVIET CARBORANE–SILOXANE STATIONARY PHASES

Name	*Chemical type of phase, functional groups*	*Solvent*	*Upper temperature limit (°C)*	*Rohrschneider constants*				
				X	*Y*	*Z*	*U*	*S*
KBS-2	Carborane–siloxane, methyl	*n*-Hexane	500	0.55	0.79	1.14	1.61	1.08
KBS-3	Carborane–siloxane, methyl	Benzene	500	0.31	0.79	0.98	1.46	0.93
KBS-2F	Carborane–siloxane, methyl, phenyl	*n*-Hexane	450	0.80	1.54	1.53	2.19	1.58
KBS-3F	Carborane–siloxane, methyl, diphenyl	Benzene	450	0.61	1.40	1.37	2.06	1.48
Dexsil-300	Carborane–siloxane, methyl	–	450	0.42	0.84	1.16	1.56	1.27

TABLE 7[118]

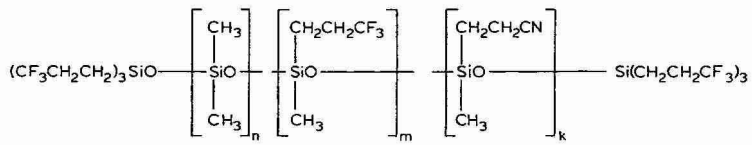
CHROMATOGRAPHIC CHARACTERISTICS OF VARIOUS STATIONARY PHASES

Stationary phase	*Working temperature range (°C)*	*McReynolds constants*				
		X	*Y*	*Z*	*U*	*S*
Dexsil-300 GC	40–450	42	84	116	156	127
Dexsil-400 GC	30–375	59	114	140	187	173
Dexsil-410 GC	20–360	85	165	169	242	180
Silbor-1	20–350	111	688	175	234	224
Quadrol	0–150	214	571	357	472	489
Hyprose SP-80	0–175	336	742	492	639	727

8. OTHER SILOXANE STATIONARY PHASES

This section deals briefly with some new siloxanes that differ in structure from those types already described, and also some copolymer materials.

Luskina *et al.*[122] proposed mixed siloxanes containing methyl, β-cyanoethyl and trifluoropropyl groups and having the general formula



where m = 0–20, n = 0–20 and k = 1–20. These stationary phases have been found to be highly selective for the separation of fluorinated and perfluorinated compounds. The maximum column temperature is 235°C.

The new high-temperature material proposed by Luskina *et al.*[123] is essentially a dimethyl polysiloxane containing 2–25% of β-ethyladamantyl groups. Examples reported included the separation of a mixture of siloxanephenanthrene derivatives at temperatures programmed to rise to 350°C and the separation of pentaphenyl ether derivatives at 325°C.

TABLE 8[118]

RETENTION INDICES OF SOME SILANE AND DIPHENYL DERIVATIVES

Chromatographed compound	*Boiling point (°C)*	*Retention index*		
		Dexsil-300	*KBS-2*	*Silbor-1*
Diphenyl	254	1497	1488	1638
m-Chlorodiphenyl	–	1616	1613	1788
p-Chlorodiphenyl	290	1708	1723	1883
Diphenyldiethoxysilane	304	1771	1808	1933
Diphenylchlorosilane	304	1865	1875	2029
Diphenyldixylenoxysilane	400	3025	3111 (400°C)	3518 (350°C)

TABLE 9[132]

EFFECT OF THE SOLID SUPPORT ON RETENTION INDICES

Column, 150 × 0.4 cm I.D., temperature, 100°C.

Solid support	*10% Squalane*						*10% Polyethylene glycol 400*					
	Hexanone-2	*Chlorobenzene*	*Pentanol-1*	*2-Methylpentanone*	*Benzonitrile*	*Nitrobenzene*	*Hexanone-2*	*Chlorobenzene*	*Pentanol-1*	*2-Methylpentanone*	*Benzonitrile*	*Nitrobenzene*
Chromosorb W	774.0	825.7	800.0	939.6	918.6	1016.9	1147.1	1347.2	1252.9	1377.0	1644.4	1745.0
Celite	747.2	826.0	763.5	902.3	904.2	1009.7	1170.0	1369.4	1275.0	1397.9	1675.8	1779.4
Anachrom	733.3	826.8	714.9	885.2	896.5	1005.2	1177.9	1384.2	1383.5	1408.8	1696.8	1801.2
Chromosorb G	732.6	826.9	714.4	835.3	896.6	1005.4	1179.0	1381.2	1284.2	1409.2	1693.3	1798.2
Gas-Chrom Q	730.9	826.8	703.9	883.2	893.1	1004.3	1181.8	1386.6	1287.6	1414.9	1702.3	1810.5
Silanized Chromosorb G	728.7	826.5	694.9	883.8	893.9	1005.1	1186.4	1390.7	1293.0	1419.1	1709.0	1817.0
Max. difference, $(I_i)_{max}$*	45	1.2	106	56	25	12	39	47	41	42	65	72

* $(I_i)_{max} = (I_i)_{max}$ (solid support A) − $(I_{i\cup min}$ (solid support B).

Kirichenko *et al.*[124] recommend, for the analysis of high-boiling compounds, siloxanes with a molecular weight of 1500–2000, containing metals in the main chain and having the general formula

$$CH_3COO[SiO(C_6H_5)_2Me]_nCOCH_3$$

where Me = Be or Cr.

Turkeltaub *et al.*[125] prepared a methylsiloxane stationary phase by polymerization and polycondensation of dimethyldichlorosilane directly on the surface of a solid support. The resulting sorbent was used to separate oligomers of dimethyl polysiloxanes with molecular weights of up to 500[126].

Yevdokimov and Lobkov[127] obtained a porous sorbent of the Porapak Q type by curing a dimethyl polysiloxane gum with a molecular weight of $5 \cdot 10^5$ under ^{60}Co gamma radiation, followed by heating of the cured product. The resulting porous polymer had a specific surface area of 20–50 m^2/g, with a size pore ranging from 35 to 550 Å and a thermal stability of 300°C.

In recent years, Vigdergauz and co-workers have published the results of various experiments with colloidal sorbents as stationary phases. Interesting results concerning the chromatography of organometallic compounds on PMS-100–Aerosil colloidal systems were reported in a review by Kirsh *et al.*[128]. For example, on a column with 6% of the PMS-100–Aerosil (2:3) colloidal system, the temperature of analysis of bis-arene complexes of chromium could be lowered from 200 to 150°C, compared with PMS-100 alone[129], which is extremely important in the analysis of unstable compounds.

9. EFFECT OF ADSORPTION PHENOMENA ON THE McREYNOLDS CONSTANTS OF STATIONARY PHASES

The McReynolds constants[45], characterizing the ability of the stationary phase to be involved in various types of intermolecular interactions, are based on the determination of the retention indices of chemical compounds of different structures. It is known[130,131] that retention indices are greatly influenced by the type and batch of the solid support. For example, Table 9[132] lists the retention indices of some polar compounds on squalane and polyethylene glycol 400, which shows that on different supports the difference between the retention indices of the same substances may be as high as 50–100 units. Table 10[133] shows the results of measurements, in five different laboratories, of the retention indices of some hydrocarbons and chloroalkanes on squalane using different batches of the solid support Chesasorb. In this instance, the spread of retention indices is large, *i.e.*, up to 38 units.

The effect of the solid support on the retention indices of McReynolds test compounds was studied earlier[44] (Table 11). In other work[132], the greatest differences were observed with non-silanized supports, especially Chromosorb, although the spread of the retention indices for compounds belonging to the same class (hexanone-2 and pentanone-2; pentanol-1 and butanol-1) was considerably smaller than that reported[132] and did not exceed 16 units.

The data suggest that it is necessary to take into account the adsorption of the sample compounds if the retention values corresponding only to their dissolution in

TABLE 10[133]

INTER-LABORATORY MEASUREMENTS OF RETENTION INDICES

Column, 200 × 0.3 cm; 6% squalane on Chesasorb; temperature, 70°C.

Compound	*Laboratory No.*					$(\Delta I)_{max}$*
	1	*2*	*3*	*4*	*5*	
cis-4-Methyl-pentene-2	561	565	563	562	–	4
Chloroform	589	595	600	597	–	11
Benzene	660	670	634	653	–	36
Dichloroethane	648	679	657	648	641	38
Heptene-1	686	688	687	684	687	4
Toluene	764	783	765	772	773	19

* $(\Delta I)_{max} = I_{max}$ (lab. A) $- I_{min}$ (lab. B).

the stationary phase are to be determined. The methods for such determinations were proposed by Berezkin[130] and are based on finding the invariant retention values from the equation

$$I_i = I_{0i} + \lambda_i/P$$

where

$$I_{0i} = 100z + 100 \log K_{1i}/K_{1z} / \log K_{1(z+1)}/K_{1z}$$

λ_i is a constant and P is the percentage of the stationary phase on the solid support (solid support weight = 100%). Note that in this equation the reciprocal of the stationary phase content may not only be the reciprocal of the percentage content of the stationary phase on the solid support, but also the reciprocal of the stationary phase volume in the column, the effective thickness of its film on the solid support or the reciprocal of the capacity ratio of the compound whose adsorption can be ignored for the chromatographic system under consideration, by virtue of its smallness.

As the relationship between the retention index and the reciprocal of the stationary phase content is usually adequately described by the above linear equation in the coordinates $I_i - 1/P$, there is every reason to use such relationships for deriving the values of I_{0i}. I_{0i} may be referred to as the limiting or invariant retention index, which places emphasis on its independence of the solid support type. I_{0i} is a function not of the retention time of the sample compounds but of the constants of its distribution. Consequently, this quantity must be more stable in the experimental environment, and its use must substantially enhance the repeatability of the experimental results. A joint experimental programme undertaken by five laboratories has corroborated this hypothesis. Table 12[133] lists the invariant retention indices for some hydrocarbons, calculated using the above equation. It can be seen that the invariant retention indices are characterized by a much better repeatability than those measured directly. For example, the maximum spread of the invariant retention indices for different compounds varies from 1 to 7 units, whereas for retention indices

TABLE 11[44]

EFFECT OF THE SOLID SUPPORT ON RETENTION INDICES

Column, 2.35 m × 0.3 cm I.D.; 20% squalane + 0.1% Span 80; temperature, 120°C.

Solid support	*Retention index*									
	Benzene		*Butanol-1*		*Pentanone-2*		*1-Nitropropane*		*Pyridine*	
	Obtained	*Difference from McReynolds constants*	*Obtained*	*Difference from McReynolds constants*	*Obtained*	*Difference from McReynolds constants*	*Obtained*	*Difference from McReynolds constants*	*Obtained*	*Difference from McReynolds constants*
Celite-545	645	+1	594	+4	631	+4	656	+4	704	+5
Chromosorb W AW	656	+3	601	+11	635	+8	662	+10	715	+16
Chromosorb W AW HMDS	652	−1	596	+6	628	+1	653	+1	706	+7
Chromosorb G (5% squalane + 0.1% Span 80)	655	+2	602	+12	634	+7	661	+9	713	+14
Chromatron N AW DMCS (batch No. 80483)	653	0	590	0	627	0	655	+3	701	+2
Chromatron N AW DMCS (batch No. 80426)	653	0	591	+1	627	0	653	+1	700	+1
Supelcoport (80–100 mesh)	653	0	591	+1	627	0	653	+1	700	+1

TABLE 12[133]

INVARIANT RETENTION INDICES, I_{0i}, FOR SOME ORGANIC COMPOUNDS ON SQUALANE AT 70°C

Compound	*Laboratory No.*					$(\Delta I_{0i})_{max}$
	1	*2*	*3*	*4*	*5*	
cis-4-Methylpentene-2	553	555	556	554	–	3
Chloroform	579	580	582	575	–	7
Benzene	640	641	642	640	–	2
Dichloroethane	600	600	605	600	601	5
Heptene-1	681	681	682	682	683	2
Toluene	750	750	750	750	749	1

measured in a traditional manner the spread is from 4 to 38 units. Hence the repeatability is improved by a factor of 4–5 when invariant retention indices are used.

A similar approach must also be used to determine the McReynolds constants. This will permit (1) the properties of the stationary phase to be defined, as opposed to those of the sorbent, and (2) improvement of the repeatability of the retention indices. It is therefore recommended that for determining the properties of stationary phases invariant Rohrschneider and McReynolds constants found from the equations

$$\frac{I_{0imn}}{100} = \frac{I_{0im} - I_{0in}}{100}$$

and

$$I_{0imn} = I_{0im} - I_{0in}$$

where I_{0imn} is the difference between the invariant maximum retention indices for a substance i and phases m and n and I_{0im} and I_{0in} are the invariant (maximum) retention indices for the substance i and stationary phases m and n, respectively, should be used. These invariant constants characterize only the properties of the stationary phase under investigation.

10. CONCLUSION

Since 1960, Soviet researchers have extensively investigated the chromatographic properties of polysiloxane materials intended for use as stationary phases. Emphasis has been placed on the development of new types of phases, and many patents have been granted. To facilitate interchangeability of the Soviet stationary phases, foreign analogues for Soviet polysiloxane stationary phases have been described.

In estimating the properties of stationary phases, and determining of important characteristics such as McReynolds/Rohrschneider constants and upper temperature limits, considerable attention has been paid to the careful purification of the phases and their conditioning, exclusion liquid chromatographic techniques being recom-

mended for establishing their properties and the transformations which they undergo. The determination of the chromatographic properties of stationary phases is based on the estimation of the contribution made by the adsorption of the sample compounds to the overall retention value, which permits the interfering effect of the solid support to be taken into consideration.

As far as future developments are concerned, more attention should be paid to ensuring uniformity of individual batches of polysiloxane stationary phases. Developments in capillary gas chromatography are also of great importance.

11. SUMMARY

Polysiloxane stationary phases developed and used in the Soviet Union are described in detail, probably for the first time in the Western literature. Materials comparable to those produced by Western specialty chromatographic suppliers are included; polysiloxanes particularly suitable for high-temperature use are available in the Soviet Union and comparable materials are not available elsewhere. The effect of adsorption on the determination of McReynolds constants of stationary phases is discussed.

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CHREV. 193

ADSORPTION CHROMATOGRAPHIC SEPARATIONS ON BUFFERED SILICA GEL

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CONTENTS

1. INTRODUCTION

The buffered silica gel separation technique has been described previously[1,2]. It is used to reduce the tailing of polar compounds in adsorption chromatography with neutral organic mobile phases. No reagents have to be added to the mobile phase, which minimizes detection problems. The separation selectivity of adsorption chromatography for isomers is well known and has been used many times to separate non-polar solutes. With buffered silica gel it is now possible to separate polar isomers by adsorption chromatography.

The use of silica gel in combination with a buffer and organic solvents has already been described by Isherwood[3] and others. An aqueous buffer solution was used to impregnate the surface of the silica gel. This layer formed a stationary phase, which converted the solutes into the ionized form where the partition favours the stationary phase. The separation mechanism is partition, because the water layer on the silica surface uses up most of the adsorption sites available and acts as a stationary phase.

By the term "buffered silica gel" we mean a silica gel that has been coated with a crystalline salt. The water used to bring this buffer salt into the pores and to distribute it evenly over the whole surface is evaporated and therefore has no influence on the chromatographic behaviour and the separation selectivity. The k' values of non-ionic compounds are the same as on a corresponding untreated silica gel system. The buffer layer affects only the peak symmetry of polar compounds and makes their elution on the adsorption chromatographic system possible. In some instances the pH of the aqueous solution of the buffer salt used to coat the silica gel can be an additional parameter for controlling the separation selectivity.

This review was presented in part as a poster at the 8th International Symposium on Column Liquid Chromatography in New York, May 1984. It enlarges on a section of a previous review on carboxylic acid separations[4].

2. EXPERIMENTAL

2.1. Apparatus

Any commercial liquid chromatograph may be used for the application of buffered silica gel columns. We used an M-6000A high-pressure pump (Waters Assoc., Milford, MA, U.S.A.) to force the mobile phase through the column. The sample was introduced with a U6K sampling loop (Waters Assoc.) and the eluent was monitored with a multi-wavelength UV detector (Pye Unicam, Cambridge, U.K.). The columns were made of glass, stainless steel or glass-lined steel and were packed in our laboratory. The technique used to slurry-pack the glass columns at 450 bar has been described previously[5]. For the preparation of the other columns a slurry of 2 g of buffered silica gel in 20 ml of *n*-hexane was packed into the column with *n*-hexane at 450 bar. During chromatography the columns were normally operated at room temperature and were not thermostabilized in any way.

2.2. Material

Different silica gels of high-performance liquid chromatographic (HPLC) quality were used as received without any further size classification of particles. All the reagents were of analytical-reagent grade (Fluka, Buchs, Switzerland) and were used without further purifications. The solvents used for packing the columns and as mobile phases were of HPLC grade (Rathburn Chemicals, Walkerburn, U.K.) and degassed with helium in an ultrasonic bath.

3. PREPARATION OF BUFFERED SILICA GEL

3.1. Batch procedure

A 150-ml volume of a 0.1 *M* aqueous solution of the buffer salt is prepared and to 50 ml of this buffer solution 5 g of silica gel are added to form a fluid slurry. The slurry is placed under vacuum (12 Torr) and immersed in an ultrasonic bath for 2 min to force the buffer solution into the pores. The slurry is then placed in a fritted disc funnel and filtered. The wet material is again added to 50 ml of buffer solution and the vacuum and ultrasonic treatment is repeated. After filtration in the fritted disc funnel the above step is repeated a third time with the final 50 ml of buffer solution.

The wet material from the last filtration is spread out on a crystallizing disc and dried at 80°C, under vacuum (12 Torr), for 20 h. The dry buffered silica gel is packed into the column as an *n*-hexane slurry, in order to prevent any of the buffer salt being washed off, under a pressure of 400–450 bar. The packed column may be equilibrated with the mobile phase or sealed and stored at room temperature for further use.

An *in situ* coating of the pre-packed silica gel columns is also possible, and permits the buffering of commercial columns. The batch procedure, however, is much more reliable and therefore preferred.

3.2. In situ preparation

The silica gel column, commercial or laboratory-packed, must first be rinsed with acetone or with any other water-miscible solvent. This intermediate solvent is then replaced completely with water. Attention to this step and the use of at least 50 column volumes of each solvent are essential for good performance of the buffered silica column. After the silica gel has been completely wetted with water, 80 column volumes of buffer solution are pumped through.

The column is then connected to a gentle stream of nitrogen and, after all the remaining buffer solution has been purged at room temperature, the column is heated at 80°C for 20 h. After cooling to room temperature it is then ready for use and can be wetted directly with the mobile phase.

4. GENERAL PROBLEM OF ADSORPTION CHROMATOGRAPHY OF POLAR SOLUTES

The adsorption chromatography of polar and/or ionic compounds on silica gel often suffers from strongly tailing peaks, which leads not only to poor separations but also to insufficient accuracy of quantitative determinations. The tailing of peaks due to a mis-match of solute polarity, adsorption activity and solvent strength can be minimized either by choosing a silica gel with appropriate properties, by adding reagents to the mobile phase to adjust its pH or by coating the silica gel surface with a buffer salt.

The pH of an aqueous silica gel slurry depends on the quantitative and qualitative distribution of hydroxyl groups on the surface. It is commonly accepted that several types of hydroxyl groups, differing in reactivity, exist on the surface of porous

TABLE I

pH VALUES OF AQUEOUS SILICA GEL SLURRIES

1.0 g of silica gel was added to 15 ml of doubly distilled water (pH 5.1) and equilibrated at room temperature for 30 min.

Silica gel	*Shape*	*Particle size (μm)*	*Pore size (nm)*	*Surface area (m^2/g)*	*pH of slurry*
Partisil 5	Angular	6	5	400	3.5
Grace silica gel	Angular	5	8	500	4.5
LiChrosorb SI 60	Angular	7	6	475	6.9
Hypersil	Spherical	5	10	200	6.9
Spherosil XOA-600	Spherical	5	3	600	6.4
Spherisorb	Spherical	5	10	180	8.9

silica gel[6]: free or isolated hydroxyl groups are assumed to be separated by a sufficient distance to prevent the formation of hydrogen bonds, and bound or paired hydroxyl groups, which can interact via hydrogen bonding. The paired hydroxyl groups are considered to be more reactive than the isolated groups and to play the dominant role in adsorption.

The pH of the aqueous silica slurry is a useful parameter in choosing the most suitable adsorbent for a given separation problem. The pHs of different silica gels

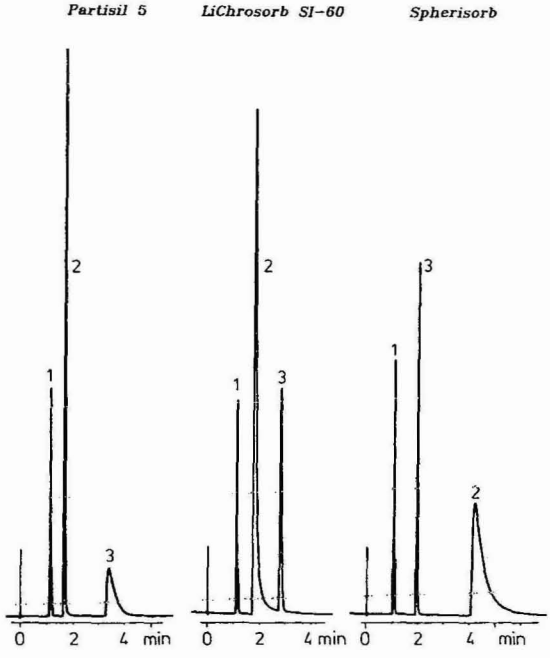

Fig. 1. Mixture of methyl benzoate (peak 1), methyl 4-hydroxybenzoate (peak 2) and methyl 4-aminobenzoate (peak 3) chromatographed on various untreated silica gels. Column: 250 × 3 mm I.D., operated at room temperature. Mobile phase: 35% *n*-hexane in diethyl ether. Flow-rate: 1.5 ml/min. Detection: UV absorption at 254 nm.

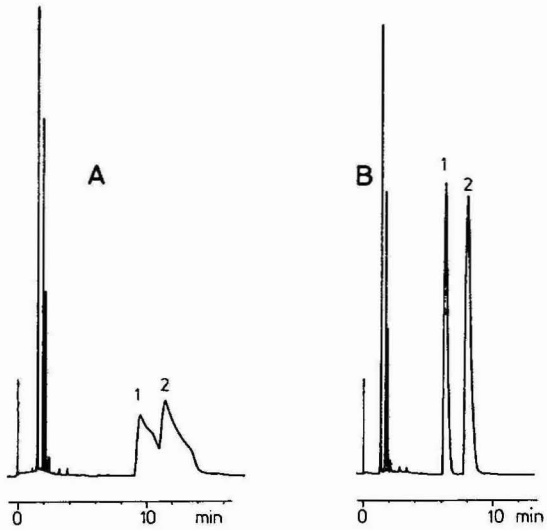

Fig. 2. Comparison of the separations of *cis*- (peak 1) and *trans*-geranic acid (peak 2) on untreated (A) and buffered (B) silica gel. Columns: both 250 × 3 mm I.D., operated at room temperature. Mobile phases: both 10% diethyl ether in *n*-hexane. Flow-rates: both 1.0 ml/min. Detection: UV absorption at 254 nm.

are listed in Table 1. They vary between 3.5 and 8.9 owing to differences in the surface chemistry of the silica gel resulting from the manufacturing process. Fig. 1 shows a practical example: acidic solutes give the best results with acidic silica gels, whereas basic compounds elute without tailing on basic silica gels. Non-ionic compounds are not influenced in their behaviour by the nature of the silica gel.

Adjusting the pH of the mobile phase by adding appropriate reagents in small amounts may also reduce tailing. The reagents should be of approximately the same polarity as the mobile phase, otherwise they will be adsorbed on the silica gel and form a stationary phase that influences not only the polar and ionic solutes in their chromatographic behaviour, but also non-ionic and non-polar compounds. Reagents in the mobile phase may also react with the solutes and give misleading results. In semi-preparative and preparative separations they may hinder the complete evaporation of the mobile phase from the collected fraction.

5. GENERAL ADVANTAGES OF BUFFERED SILICA GEL

When the pH range of the commercially available silica gels is inadequate to give a tail-free elution and reagents in the mobile phase have to be avoided for other reasons, then the use of buffered silica gel is the only method for adsorption chromatographic separations of polar solutes. The crystalline buffer on the surface of the adsorbent does not influence the chromatographic behaviour of non-polar solutes, nor does it limit the adsorption mechanism. As the buffer salt is virtually insoluble in the mobile phase, this separation system can be used for preparative separations like any normal silica gel system. The stability is comparable to that of untreated silica gel. The main advantage, however, is its potential to be used for the separation of polar solutes in the adsorption chromatographic mode, as shown in Fig. 2, with a neutral mobile phase.

6. THE BUFFER LAYER

6.1. Nature of the buffer

The term "buffered" may be somewhat misleading, as the modifying agent need not be a buffer salt in the usual sense. Both organic acids and inorganic salts have been used successfully. No change in retention behaviour of solutes can be observed on changing from one cation to another of the salt used to coat the silica gel.

The restriction on the choice of the buffer salt is its solubility in organic solvents, which should be as low as possible to prevent leaching from the column. The lower the solubility of the salt in the organic mobile phase, the better is the stability of the chromatographic system. Mostly we used citrate, phosphate and borate buffers to create pHs between 2 and 12.

6.2. Concentration of the buffer

The concentration of the buffer salt on the silica gel surface is controlled by its concentration in the aqueous solution used to coat the silica gel. The higher this concentration, the more salt will remain on the silica gel surface. A 0.1 *M* solution leads to a coating of approximately 1.5–2.5% by weight on silica gels with a specific surface area of about 400 m^2/g.

Concentrations of the buffer solution below 0.05 *M* lead to inadequate coverage of the surface. The tailing of peaks is not fully eliminated for very polar solutes. This lower limit of concentration is independent of the nature of the buffer salt.

Concentrations of buffer in the aqueous solution higher than 1 *M* lead to blocking of pores and thereby to a decrease in surface area. This results in a lower plate number of the column and in shorter retention times in comparison with normally treated columns. The chromatographic resolution of solutes is lower, although there are no tailing bands observable.

7. ROLE OF THE pH IN THE BUFFERED SILICA GEL SYSTEM

The only relationship between the buffer salt and the chromatographic behaviour of the solutes is in the pH of the aqueous solution of the salt used to coat the surface of the silica gel. All the pH values mentioned refer to the pH of this solution, as neither in the column nor in the mobile phase is there any means of characterizing the acidity of the silica gel. The pH of the aqueous solution has to be adjusted to a chosen value by mixing crystalline salts, acids or bases. Any liquids would easily be washed off and thereby alter the behaviour of the buffered silica gel system.

7.1. Buffer for acidic solutes

The pH of the aqueous solution used to buffer the silica gel has to be low for acidic solutes. In general, the pH of the solution should not be higher than the pK_a value of the solute to be chromatographed (Fig. 3A). Lower pHs will not influence the peak shape but may result in changes in the retention selectivity owing to hydro-

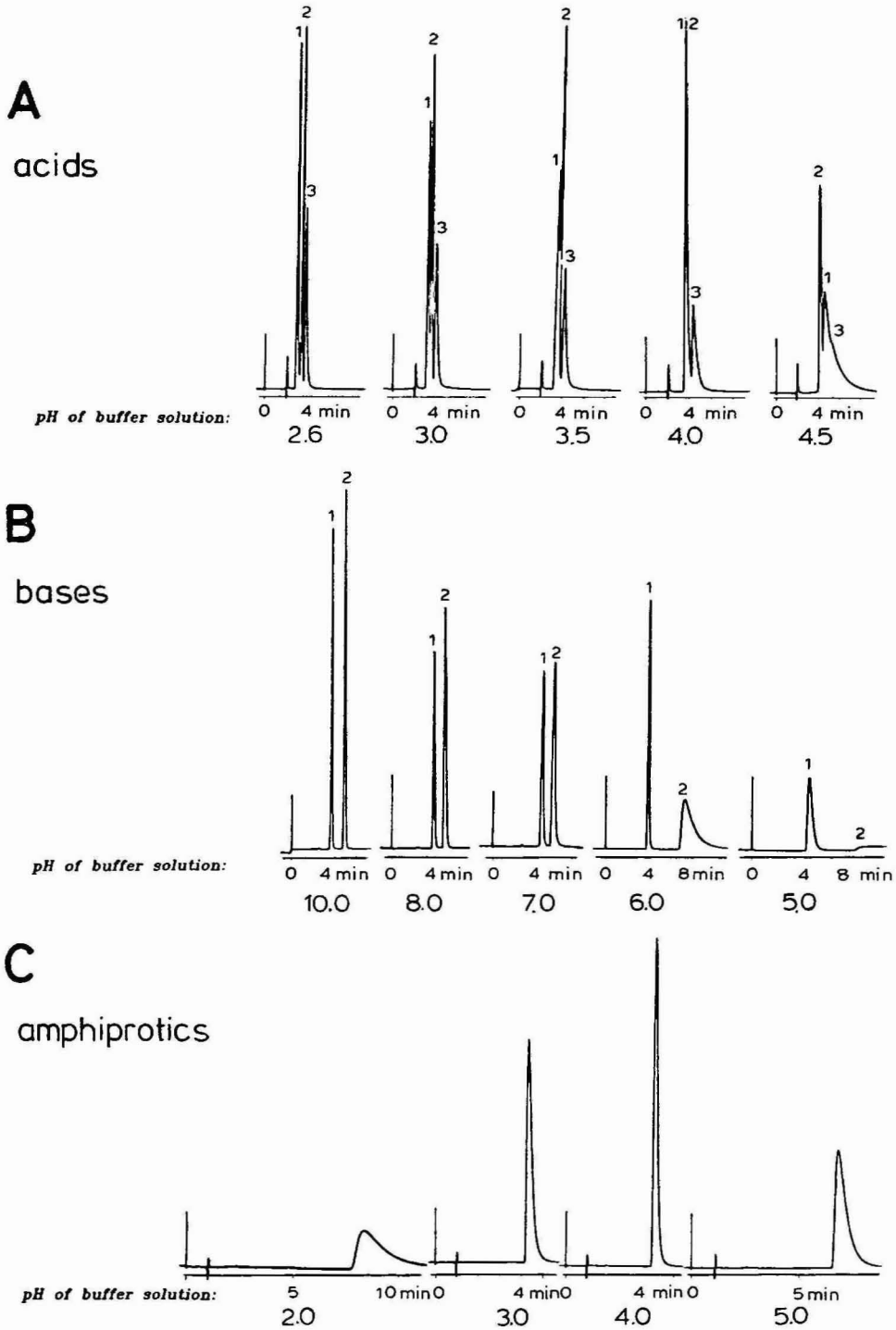

Fig. 3. Role of pH in the buffered separation system. Column: Grace silica gel, 80 Å, 5 μm, 0.1 *M* buffered, 250 × 3 mm I.D., operated at room temperature. Mobile phase: 30% *n*-hexane in diethyl ether. Flow-rate: 1.5 ml/min. Detection: UV absorption at 280 nm. (A) Peaks: 1 = 2,3-dihydroxybenzoic acid; 2 = 2,4-dihydroxybenzoic acid; 3 = 2,5-dihydroxybenzoic acid. (B) Peaks: 1 = aniline; 2 = quinoline. (C) Solute: 4-aminobenzoic acid, pK_a = (1) 2.3 and (2) 4.9.

gen bonding. This possibility of using the pH to select the best separation conditions will be further investigated.

The behaviour of acidic solutes on buffered silica gel cannot be influenced by the nature of the buffer salt. It may be a fruit acid or an inorganic acidic salt such as sodium hydrogen sulphate. We obtained very good results by using citrate–phosphate buffers. Citric acid and disodium hydrogen phosphate can be mixed to adjust any pH in the range 2.2–7.8. For pHs below 2.2 citric acid, oxalic acid and sodium hydrogen sulphate have been used successfully.

7.2. Buffer for basic solutes

For tail-free elution of polar basic compounds the silica gel has to be coated with a basic salt. The pH of the buffer solution used should be higher than the pK_a of the solutes (Fig. 3B). Usually we use a sodium borate buffer to adjust pHs between 8 and 12.

It is generally accepted that above pH 9 the solubility of amorphous silica increases expoonentially[6]. As the time of contact between the aqueous basic buffer solution and the silica gel during the preparation step is short, and the coating is carried out at room temperature, no dissolution of silica gel could be observed by negative influences on the chromatographic performance characteristics.

Silica gel columns buffered at pH 10 and 12 showed the same stability and lifetime as untreated silica gel columns. The non-aqueous, non-polar mobile phase is not able to dissolve either buffer salt or silica gel and therefore the stability of the chromatographic performance is excellent; neither the separation selectivity nor the plate number change.

Whereas acidic buffered silica gel could be replaced by a system containing an acidic mobile phase, the addition of a base to adjust the pH of the mobile phase to 10–12 is not feasible. It would slowly dissolve the silica packing of the column and

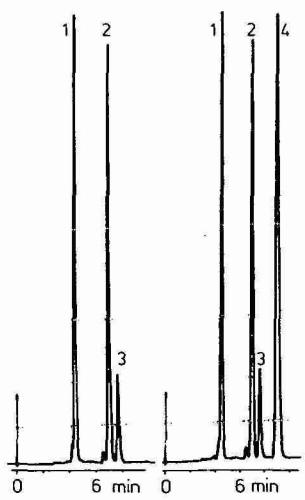

Fig. 4. Non-ionic compounds on buffered silica gel. Column: Grace silica gel, 80 A, 5 μm, 250 × 3 mm I.D., operated at room temperature. Mobile phase: 25% diethyl ether in *n*-hexane. Flow-rate: 1.0 ml/min. Detection: UV absorption at 215 nm. Peaks: 1 = lilial; 2 = lilial ketone; 3 = impurity; 4 = lilialic acid.

thereby alter the chromatographic performance of the system. Basic solutes therefore require the unique property of basic buffered silica gel.

7.3. Buffer for amphiprotic solutes

Amphiprotic compounds, which contain both acidic and basic groups, are best chromatographed on silica gel buffered at a pH equal to the isoelectric point (Fig. 3C). At too low a pH the basic groups of the molecules will lead to tailing bands and at too high a pH the acidic groups become responsible for unacceptable peak shapes.

7.4. Non-ionic and non-polar compounds on buffered silica gel

Non-ionic solutes exhibit the same behaviour on buffered silica gel as on untreated material (Fig. 4). The separation selectivity and the retention are not affected by the buffer layer. In the given example the aldehyde (lilial), the ketone and the impurities are separated as well on buffered silica gel as on the untreated adsorbent. Lilialic acid, however, is not eluted at all from normal silica gel, but can be detected as a symmetrical peak on buffered silica gel systems.

A comparison of the chromatographic behaviour of non-ionic solutes on untreated and buffered silica gel indicates that all the adsorption sites on buffered silica gel are still available for the chromatography and are not occupied by the buffer salt.

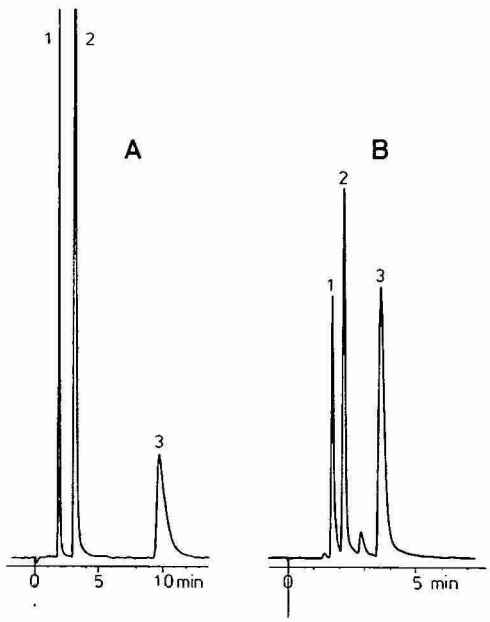

Fig. 5. Separation of polar isomers. Column: Partisil 5, 7 μm, buffered to pH 1.1 (tartaric acid), 250 × 3 mm I.D., operated at room temperature. Mobile phase: (A) 50% diethyl ether in *n*-hexane; (B) 100% diethyl ether. Flow-rate: 1.0 ml/min. Solutes: (A) trihydroxybenzoic acids, 1 = 2,3,4-trihydroxybenzoic acid, 2 = 2,4,6-trihydroxybenzoic acid, 3 = 3,4,5-trihydroxybenzoic acid; (B) flavones, 1 = kaempferol, 2 = galangin, 3 = fustin.

8. APPLICATIONS OF BUFFERED SILICA GEL SYSTEMS

The possible applications of buffered silica gel separation systems are numerous and therefore only a few examples can be given.

8.1. Separation of isomers

Adsorption chromatography is the best separation technique for isomers. In a previous paper[4] we showed the separation of dihydroxybenzoic acid isomers and compared it with the less successful results obtained with other types of separation systems. Another example is given in Fig. 5A. The complete separation of the dihydroxybenzoic acid isomers is possible on buffered silica gel with a neutral mobile phase. These examples show clearly that the separation power of adsorption chromatography is now available for polar compounds.

8.2. Separation of flavones

The solubility of some flavones in diethyl ether is good enough for them to be chromatographed with an organic mobile phase on buffered silica gel (Fig. 5B). The advantage of the buffered separation system is the compatibility with organic extracts from plants and fruits, and the possibility of a facilitated evaporation of the mobile phase for further identification of the components of the collected fractions. As flavone glucosides are insoluble in most organic solvents a pre-separation is achieved at the extraction stage. Aqueous separation systems for flavones, flavanones and flavanoids have been described by several workers[7–9].

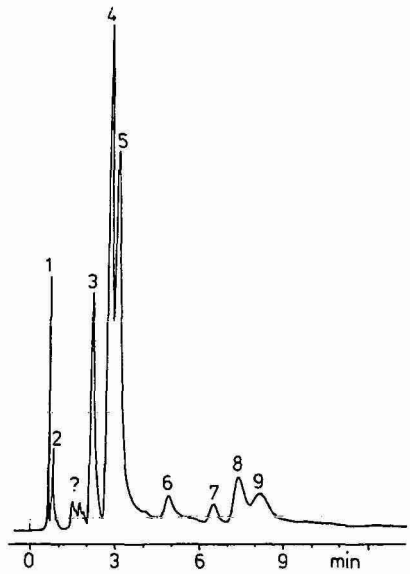

Fig. 6. Separation of hop acids in a beer extract. Column: LiChrospher SI 100, 5 μm, buffered to pH 2.6 (citrate), 250 × 3 mm I.D., operated at room temperature. Mobile phase: 10% diethyl ether in *n*-hexane. Flow-rate: 1.0 ml/min. Solutes: 1 = solvent; 2 = β-acids; 3 = *cis*-ad-iso-α-acid; 4 = *cis*-co-iso-α-acid; 5 = *cis*-*n*-iso-α-acid; 6 = *trans*-ad-iso-α-acid; 7 = α-acid; 8 = *trans*-co-iso-α-acid; 9 = *trans*-*n*-iso-α-acid.

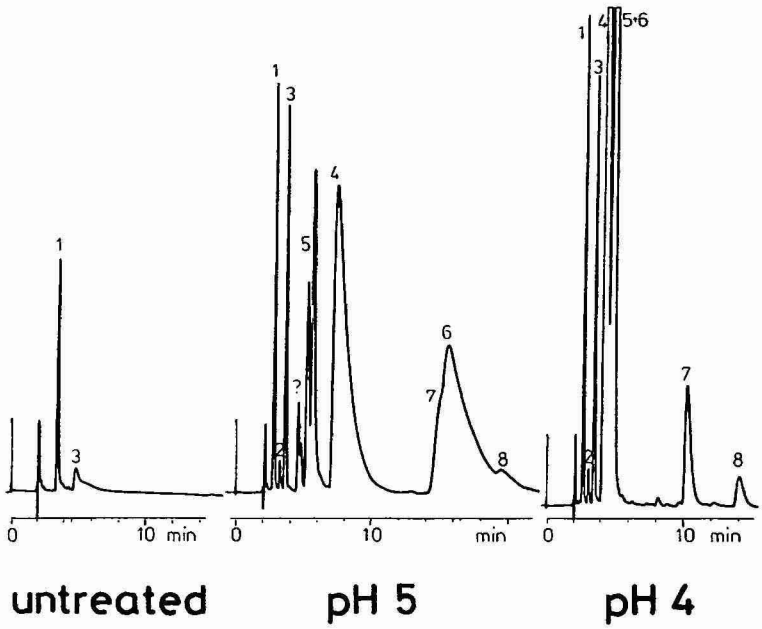

Fig. 7. Separation of polar fraction of ylang-ylang oil. Column: Grace silica gel, 80 Å, 5 μm, 250 × 3 mm I.D., operated at room temperature. Mobile phase: 34% diethyl ether in *n*-hexane. Flow-rate: 1.5 ml/min. Detection: UV absorption at 210 nm. Peaks: 1 = methyl salicylate; 2 = ?; 3 = isoeugenol; 4 = eugenol; 5 = *p*-cresol; 6 = benzoic acid; 7 = vanillin; 8 = acetic acid.

8.3. Hop acids in beer and hop products

The bitter acids in hop and beer form a complex mixture of closely related compounds. Separations have been accomplished by counter-current distribution and by thin-layer, paper and ion-exchange chromatography. Partition chromatographic systems on C_{18} reversed-phase packings have been described by Verzele and co-workers[10,11]. The separation on buffered silica gel gives more information in a shorter time, because the homologues and their isomers are separated[12] (Fig. 6).

8.4. Polar fractions of natural oils

The analysis of flavour compounds in oils and extracts of plants, flowers and fruits is usually carried out by gas chromatography. The acid fractions of these plant extracts or oils, however, lend themselves better to HPLC for separation. Reversed-phase and other HPLC methods have been described[4,13]. The buffered silica gel system may be complementary to the others. Fig. 7 shows the possibilities: by choosing a suitable pH, the separation selectivity can be changed as required. At higher pHs some strongly acidic solutes do not elute and hence can be suppressed.

8.5. Separation of 1,3-diketones

Fig. 8 shows the separation of the impurities from Parsol 1789 (a sun-screen

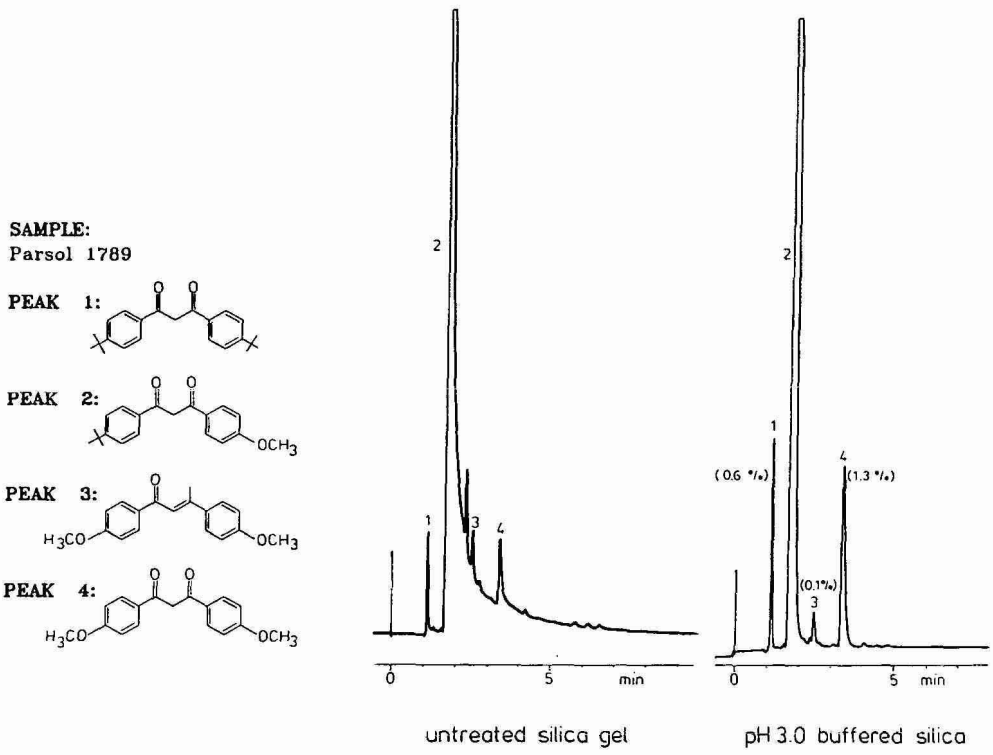

Fig. 8. Determination of small amounts of impurities of 1,3-diketone. Column: Grace silica gel, 80 Å, 5 μm, 250 × 3 mm I.D., room temperature. Mobile phase: 20% tetrahydrofuran in *n*-hexane. Flow-rate: 1.5 ml/min. Detection: UV absorption at 280 nm. Sample: Parsol 1789.

product). These 1,3-diketones tend to tail owing to the formation of hydrogen bonds, even when using apolar, neutral mobile phases. On buffered silica gel the tailing can be reduced and the determination of small amounts of the impurities is possible.

8.6. Preparative and semi-preparative separations

For preparative separations of polar compounds, the buffered silica gel systems are preferred to reversed-phase systems, because the mobile phase can easily be removed from the collected fraction as it is only a mixture of low-boiling organic solvents and free of any reagents (buffers, acids, etc.). The collection of peaks from the analytical column for further identification purposes is also facilitated.

8.7. HPLC–MS coupling

In direct liquid interfacing of HPLC with MS the flow-rate of the mobile phase has to be adjusted in order to reduce the amount of gas evolved from the complete vaporization of the solvent to the maximum allowed level for operation of the mass spectrometer under high vacuum. A flow-rate of 1 ml/min of water will give about 1200 ml/min of water vapour. By using the buffered silica gel system for LC–MS

coupling of polar compounds, with *n*-hexane and diethyl ether as the mobile phase, the flow-rate may be five times the maximum allowed value for a reversed-phase system, because 1 ml/min of *n*-hexane will give only about 180 ml/min of *n*-hexane vapour.

9. LIMITATIONS

The only disadvantages of the buffered silica gel system is the limited choice of the mobile phase composition. The buffer salt should not dissolve in the mobile phase. However, we successfully used diethyl ether–ethanol–dimethyl sulphoxide (1000:125:30) on citric acid-coated silica gel to separate highly polar compounds such as polar PTH-amino acids, gallic acids, ascorbic acid, tartaric acid and malic acid. Under these conditions very small amounts of buffer could be found in the eluent and the lifetime of the column was reduced to only about 3–4 weeks. By using *n*-hexane–diethyl ether as the mobile phase the stability and the column lifetime are comparable to those of normal silica gel columns.

10. CONCLUSIONS

The buffered silica gel separation systems allow tail-free elution of polar solutes with neutral, organic mobile phases. They have the separation power of adsorption chromatography and show the same chromatographic behaviour as untreated silica gel for non-polar solutes. The stability of buffered systems is comparable to that of untreated silica gel systems provided that the buffer salt is virtually insoluble in the mobile phase. Gradient elution and flow programme can be used as with normal silica gel systems. The buffered silica gel facilitates the semi-preparative and preparative isolation of polar solutes and the direct liquid inlet coupling of HPLC with mass spectrometric detectors.

11. ACKNOWLEDGEMENTS

I am very grateful to Danièle Gubler and Peter Kunz for their assistance and collaboration.

12. SUMMARY

HPLC separations of polar solutes, by adsorption chromatography on silica gel, is often hindered by tailing of the bands. By treating the silica gel with a buffer salt, insoluble in the mobile phase, an environment is created on the surface of the adsorbent that permits tail-free elution of very polar compounds.

The influence of the nature of the buffer salt and of its concentration and pH has been demonstrated. Buffered silica gel is not only an alternative to reversed-phase chromatography but may also be superior to it for the separation of isomers or for semi-preparative separations.

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CHREV. 190

WEAK ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PEPTIDES

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CONTENTS

1. INTRODUCTION

During the past decade, high-performance liquid chromatography (HPLC) has become a powerful tool for peptide separations. The reversed-phase mode of HPLC (RP-HPLC) has been the most popular and broadly used technique for this purpose (for reviews, see refs. 1 and 2). Ion-exchange HPLC has also been employed for peptide separations, although to a lesser extent (for reviews, see refs. 3 and 4).

Recently, a novel method was introduced for the separation of underivatized dipeptides, including resolution of sequence isomeric and diastereomeric dipeptides by weak anion-exchange HPLC (WAE-HPLC)[5]. For this purpose, a commercially available column, *i.e.* MicroPak AX-10 (Varian, Walnut Creek, CA, U.S.A.), was used. The MicroPak AX-10 is a weak anion-exchange bonded stationary phase carrying the functional group $-CH_2-CH_2-CH_2-\overset{+}{N}H_2-CH_2-CH_2-\overset{+}{N}H_3$, which is covalently bonded to fully porous silica (LiChrosorb Si-60). This stationary phase with an exchange capacity of 2 mequiv./g (5 mequiv. per column of the size 30 × 0.4 cm) had been introduced previously for simultaneous analysis of nucleobases, nucleosides and

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nucleotides[6], and is used for the separation and sequencing of sequence isomeric deoxypentanucleotides[7].

Peptides are usually not retained well on the weak anion-exchanger using aqueous buffers as mobile phases. However, addition of an organic modifier such as acetonitrile to the buffer increases peptide retention. Mixtures of triethylammonium acetate (TEAA) buffer and acetonitrile were used as the eluent for separation of dipeptides[4]. This mobile phase has minimal absorbance at wavelengths in the range of 210 to 225 nm allowing gradient elution and sensitive detection of peptides. Moreover, the TEAA buffer is volatile and thus facilitates the easy recovery of eluted peptides for further use and study. Gradient analysis is carried out by increasing the amount of the buffer in the eluent.

The purpose of this paper is to review the applications of this method to peptide separations.

2. SEPARATION OF DIPEPTIDES

Efficient separation of dipeptide mixtures is required in the sequence analysis of peptides using dipeptidyl peptidases, in which enzymatic digestions coupled with the Edman degradation yield overlapping sets of dipeptides[8]. The identification of the released dipeptides is used to reconstruct the sequence of the peptides. Dipeptides have been separated by a variety of techniques, including cation-exchange HPLC[9], and RP-HPLC[10,11].

Fig. 1 shows the separation by WAE-HPLC of a mixture of sixteen selected

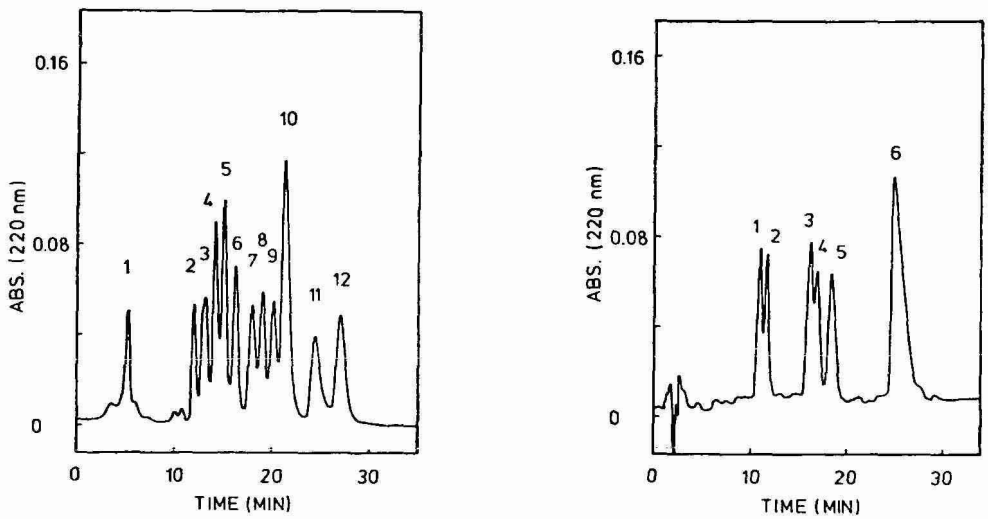

Fig. 1. Separation of some selected dipeptides. Column, MicroPak AX-10 (10 μm), 30 × 0.4 cm. Temperature, 40°C. Eluent, 0.01 *M* TEAA (pH 4.3)–acetonitrile (32:68). Flow-rate, 1 ml/min. Peaks: 1 = L-Arg–L-Phe; 2 = L-Leu–L-Leu; 3 = Gly–L-Ile and L-Leu–L-Trp; 4 = L-Ala–L-Ile; 5 = L-Trp–Gly; 6 = L-Trp–L-Phe; 7 = L-Val–L-Val and L-Ala–L-His; 8 = L-Trp–L-Ala; 9 = L-Ala–L-Thr and L-Met–L-Met; 10 = Gly–Gly and L-Phe–L-Phe; 11 = L-Ser–L-Phe; 12 = L-Tyr–L-Tyr.

Fig. 2. Separation of some dipeptides containing acidic amino acids. Column as in Fig. 1. Temperature, 40°C. Eluent, 0.01 *M* TEAA (pH 3.1)–acetonitrile (60:40). Flow-rate, 1.5 ml/min. Peaks: 1 = L-Ala–L-Glu; 2 = Gly–L-Glu; 3 = α-L-Glu–L-Ala; 4 = L-Ala–L-Asp; 5 = Gly–L-Asp; 6 = γ-L-Glu–L-Leu.

dipeptides[5]. The absorption profile represents a resolution of twelve peaks. The conditions used for the separation shown in Fig. 1 do not elute acidic dipeptides containing aspartate and glutamate residues; these species can be eluted by reducing the pH of the aqueous buffer as shown in Fig. 2.

2.1. Sequence isomeric dipeptides

Resolution of sequence isomeric dipeptides is important in peptide sequencing by dipeptidyl peptidases. An isocratic separation of a six-pair mixture of isomeric dipeptides by WAE-HPLC is shown in Fig. 3; another six-pair mixture required use of a gradient to elute all components in reasonable time (Fig. 4). Except DL-Leu–DL-Ala, and DL-Ala–DL-Ser, the dipeptides in these mixtures were in L,L-configuration. DL-Leu–DL-Ala and DL-Ala–DL-Ser each gave two peaks (peaks 1 and 4 in Fig. 3 and peaks 6 and 9 in Fig. 4, respectively). In each case, the peak with the longer retention time most probably represents the L,L-form as discussed below in separation of diastereomers.

The elution order of a given pair of sequence isomeric dipeptides appears to obey certain rules. The dipeptide with the most hydrophobic residue as the C-terminal amino acid elutes first. For dipeptides containing only aliphatic amino acids, the dipeptide having a shorter side chain in the N-terminal amino acids elutes first. These rules, of course, cannot be generalized without examining all possible pairs of sequence isomeric dipeptides.

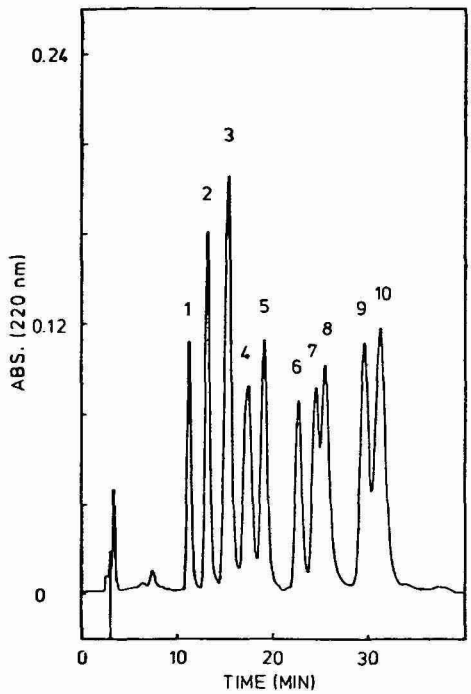

Fig. 3. Separation of sequence isomeric dipeptides. Column details in Fig. 1. Peaks: 1 = DL-Leu–DL-Ala; 2 = Gly–L-Phe; 3 = L-Ala–L-Leu, Gly–L-Met and L-Ala–L-Phe; 4 = Gly–L-Try and DL-Leu–DL-Ala; 5 = L-Ala–L-Tyr; 6 = L-Phe–Gly; 7 = L-Tyr–Gly; 8 = L-Met–Gly; 9 = L-Phe–L-Ala; 10 = L-Tyr–L-Ala.

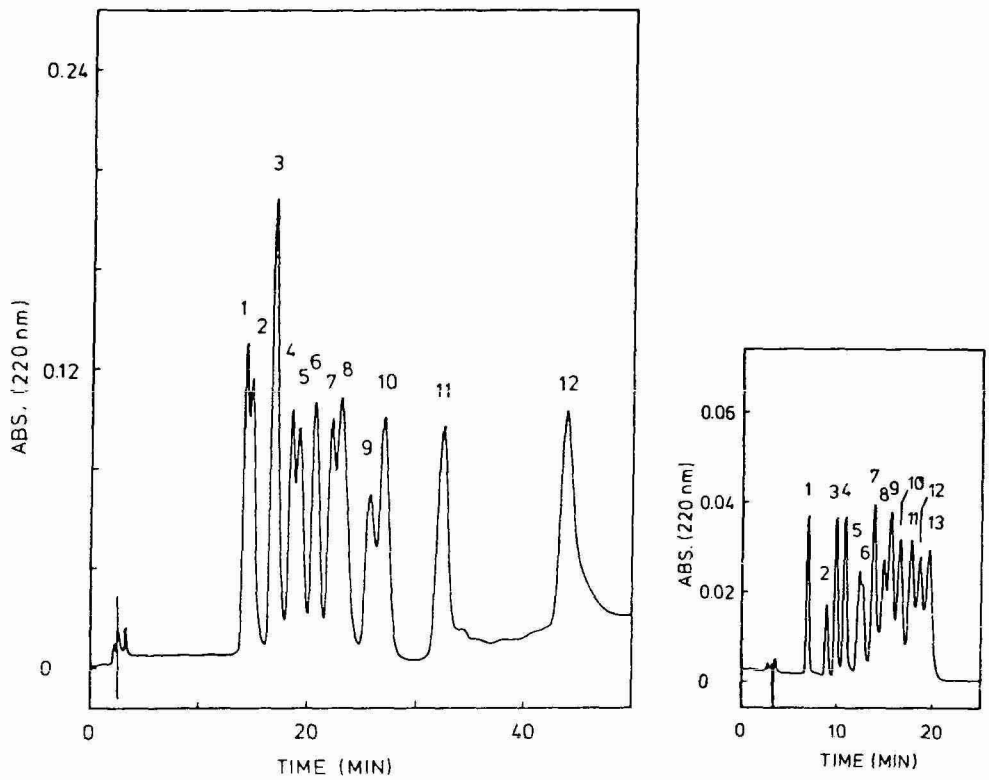

Fig. 4. Separation of sequence isomeric dipeptides. Column as in Fig. 1. Temperature, 50°C. Eluent: A, acetonitrile; B, 0.01 *M* TEAA (pH 4.3), gradient program: step 1, isocratic with 70% A and 30% B for 25 min; step 2, linear gradient of 0.4% B per min. Flow-rate, 80 ml/h. Peaks: 1 = Gly–L-Leu; 2 = L-Leu–Gly; 3 = L-Ala–L-Met and Gly-L–Val; 4 = L-Val–L-Phe; 5 = L-Val–Gly; 6 = DL-Ala–DL-Ser; 7 = L-Ala–Gly; 8 = Gly–L-Ala; 9 = DL-Ala–DL-Ser; 10 = L-Phe–L-Val; 11 = L-Met–L-Ala; 12 = L-Ser–L-Ala.

Fig. 5. Separation of diastereomeric dipeptides. Column as in Fig. 1. Temperature 45°C. Eluent, 0.01 *M* TEAA (pH 4.3)–acetonitrile (35:65). Flow-rate, 1 ml/min. Peaks: 1 = DL-Leu–DL-Phe; 2 = DL-Ala–DL-Phe; 3 = DL-Leu–DL-Ala; 4 = DL-Ala–DL-Val; 5 = DL-Leu–DL-Phe; 6 = L-Ala–L-Phe; 7 = DL-Ala–DL-Ala and DL-Ala–DL-Val; 8 = DL-Leu–DL-Ala; 9 = DL-Ala–DL-Ser; 10 = DL-Ala–DL-Asn; 11 = DL-Ala–DL-Ala; 12 = DL-Ala–DL-Ser; 13 = DL-Ala–DL-Asn.

2.2. Diastereomeric dipeptides

A number of DL,DL-peptides were shown to be separable by WAE-HPLC into their diastereomers[5] as Fig. 5 illustrates. All DL,DL-dipeptides examined except DL-Ala–DL-Phe were resolved into two peaks. The DL-Ala–DL-Phe mixture apparently does not contain the L,L-configuration because L-Ala–L-Phe shows a longer retention time (peak 6 in Fig. 5) than DL-Ala–DL-Phe (peak 2 in Fig. 5). Since individual standards of all four diastereomers were not available for these dipeptides, peak assignments were based on the elution order of the available four Ala–Ala diastereomers. In this case, the D,L- and L,D-isomers elute early as a single peak (peak 7, Fig. 5), while the D,D- and L,L-isomers coelute later (peak 11, Fig. 5). This elution order of diastereomeric dipeptides is the same found for these compounds on a cation-exchanger[11]; but the opposite of that observed in RP-HPLC[12].

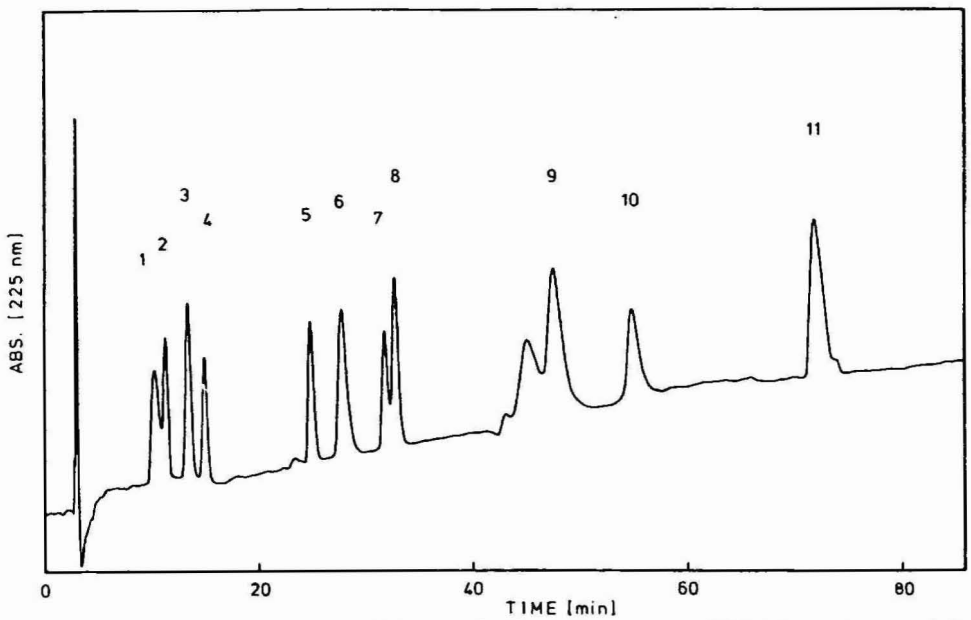

Fig. 6. Separation of various peptides. Column as in Fig. 1. Temperature, 30°C. Eluent: A, acetonitrile; B, 0.01 *M* TEAA (pH 6.0), gradient program: linear starting from 25% B at a rate of 1% B per min. Flow-rate, 1 ml/min. Peaks: 1, somatostatin; 2, proctolin; 3, neurotensin; 4, Met-enkaphalin; 5, bradykinin potentiator c; 6, Lys–Glu–Thr–Tyr–Ser–Lys; 7, α-endorphin; 8, EAE-peptide; 9, glucagon; 10, ribonuclease s-peptide; 11, IgE-peptide.

3. SEPARATION OF PEPTIDES

The WAE-HPLC method has recently been applied to the separation of longer peptides with a slight change in the pH of the TEAA buffer[13]. The separation by this method of a multicomponent peptide mixture is shown in Fig. 6. The peak identification is given in the legend of Fig. 6. A good peak symmetry was obtained for all peptides in this mixture. The amino acid analysis of eluted peptides confirmed the authenticity of peaks in Fig. 6 (for sequences, see ref. 13).

3.1. Acidic peptides

Peptides containing a number of acidic amino acid residues with no compensating basic residues either did not elute from the column or had unacceptably long retention times when the elution conditions described in Fig. 6 were used. Such acidic peptides were chromatographed using an isocratic flow of dilute formic acid instead as the eluent[13], as is shown in Fig. 7. Both ribonuclease s-peptide (RSP) and IgE-peptide (peaks 1 and 2, respectively) could also be eluted with the solvent system described in Fig. 6. Although RSP contains three acidic amino acid residues (for sequences of peptides in Fig. 7, see ref. 13), it elutes much earlier than the other peptides in this mixture. This might be due to the compensation of acidic residues by the basic amino acids. Dilute formic acid solutions used as eluent for these separations are compatible with the column and pumps used and also volatile, allowing

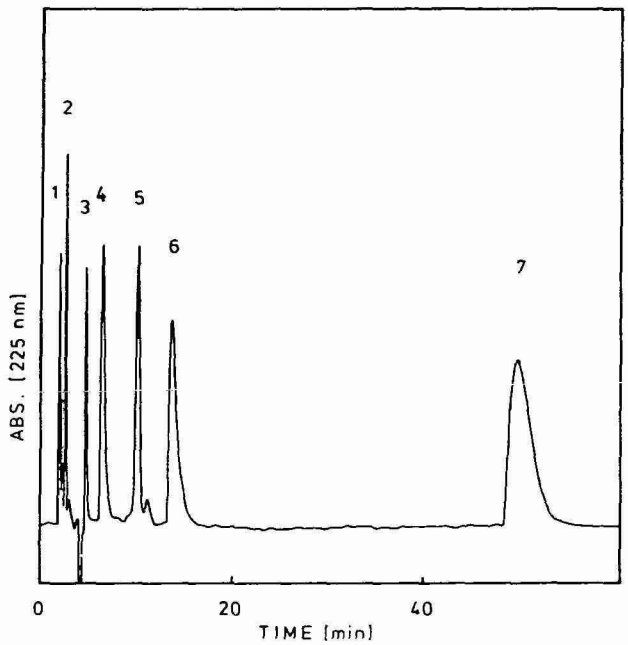

Fig. 7. Separation of some acidic peptides. Column as in Fig. 1. Temperature, 60°C. Eluent, 0.04 *M* formic acid (pH 2.6). Flow-rate, 1 ml/min. Peaks: 1, ribonuclease s-peptide; 2, IgE-peptide; 3, glutathionie (oxidized form); 4, Phe–Leu–Glu–Glu–Ile; 5, delta sleep-inducing peptide; 6, γ-Glu–Leu; 7, γ-Glu–Glu.

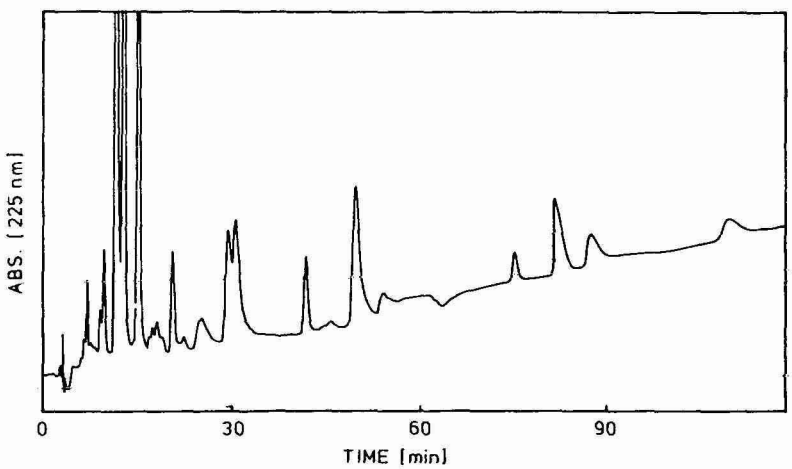

Fig. 8. Separation of a tryptic digest of horse heart cytochrome *c*. Column details as in Fig. 6 except gradient program: linear starting from 25% B at a rate of 0.6% B per min to 50% B, then 4.5% B per min to 100% B. Amount of injection, *ca.* 10 nmol of cytochrome *c*.

easy recovery of eluted peptides. As with the TEAA buffer, peptide recoveries were found to be 80% or greater[13].

3.2. Enzymatic digests of peptides

Enzymatic digestion is frequently employed in sequence analysis of peptides and proteins. For this reason, the WAE-HPLC method was also applied to separation of peptides resulting from tryptic digestion of some proteins[13]. Fig. 8 shows the separation of a tryptic digest of horse heart cytochrome *c*. The elution became isocratic at 100% buffer after 60 min, and another four peaks were observed. The number of the peaks detected in Fig. 8 corresponds closely to the number of fragments expected from the known sequence of horse heart cytochrome *c* upon trypsin digestion[14]. Assignment of the peptide fragments in Fig. 8 was, however, not carried out. Since some digestion products contain a number of acidic amino acid residues, the separation of the digestion mixture was also undertaken using the conditions given in Fig. 7. As Fig. 9 shows, the majority of the fragments had no or little retention using formic acid as eluent; however, four other peptides with significant retention were observed.

Fig. 10 shows the separation of a tryptic digest of reduced and alkylated lysozyme[13]. Again, the number of major peaks detected corresponded closely to the number of fragments expected from tryptic digestion of lysozyme[15]. In this case, no additional peptide fragments were observed when formic acid was used for elution.

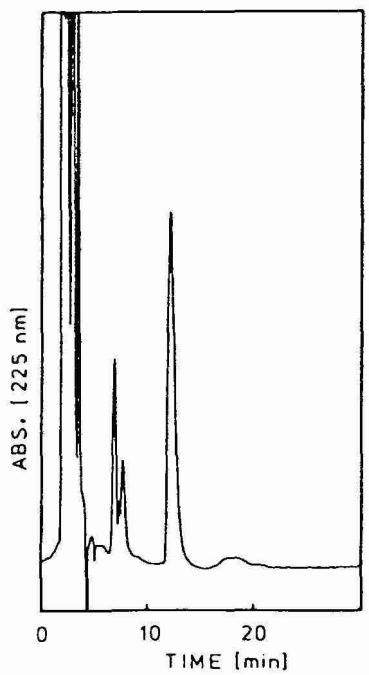

Fig. 9. Separation of a tryptic digest of horse heart cytochrome *c*. Column details as in Fig. 7. Amount of injection *ca.* 10 nmol of cytochrome *c*.

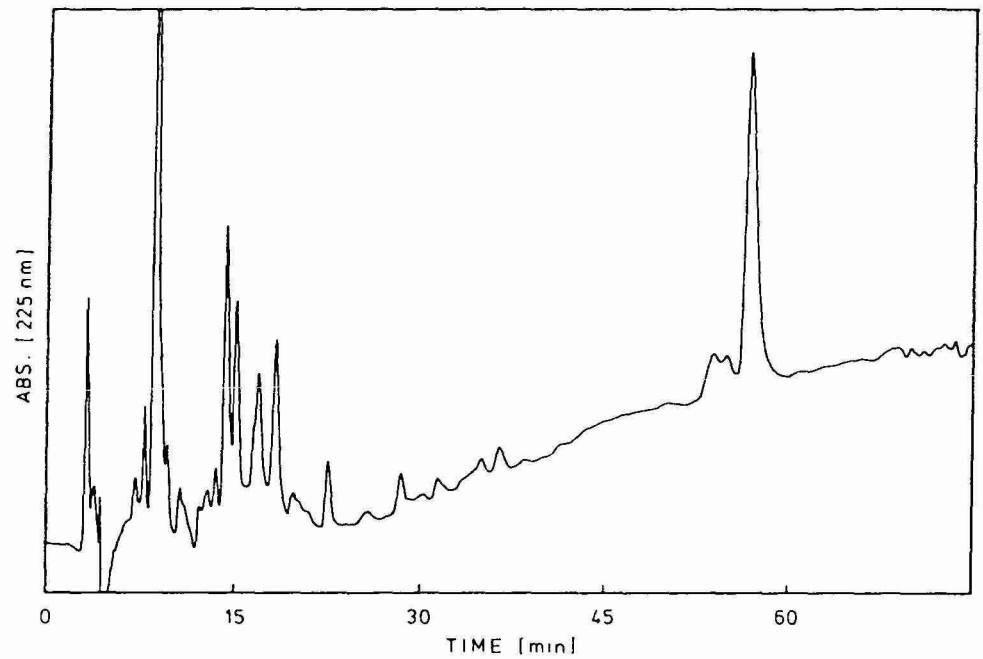

Fig. 10. Separation of a tryptic digest of reduced and alkylated lysozyme. Column details as in Fig. 6 except temperature: 40°C. Amount of injection *ca.* 10 nmol of lysozyme.

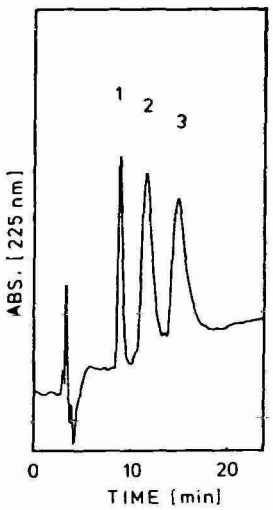

Fig. 11. Separation of several bradykinins. Column details as in Fig. 6 except gradient program: linear starting from 25% B at a rate of 1.7% B/min. Amount of injection, *ca.* 2 μg per peptide. Peaks: 1, bradykinin; 2, Met,Lys-bradykinin; 3, Lys-bradykinin.

4. SEPARATION OF CLOSELY RELATED PEPTIDES

The WAE-HPLC method was also shown to be useful in separating closely related peptides. Some applications are discussed below.

4.1. Bradykinins

Separation of several bradykinins[13] is shown in Fig. 11. Bradykinin gave a symmetrical peak (peak 1), whereas Met,Lys-bradykinin and Lys-bradykinin (peaks 2 and 3, respectively) yielded somewhat broad peaks (for sequences, see ref. 13).

4.2. Angiotensins

Angiotensins (A) are hormones with important biological activities[16,17]. These compounds are closely related peptides and differ from one another in most cases by only one amino acid residue. The WAE-HPLC method was successfully applied to separation of angiotensin[18]. Fig. 12 shows the separation of a mixture of twelve angiotensins by gradient elution. Peak identification is given in the legend of Fig. 12. Recoveries of 90 to 98% for all A's tested have been obtained[18]. Two A's (peak 6) were not resolved at that temperature. They could, however, be separated by elevation of column temperature as discussed below.

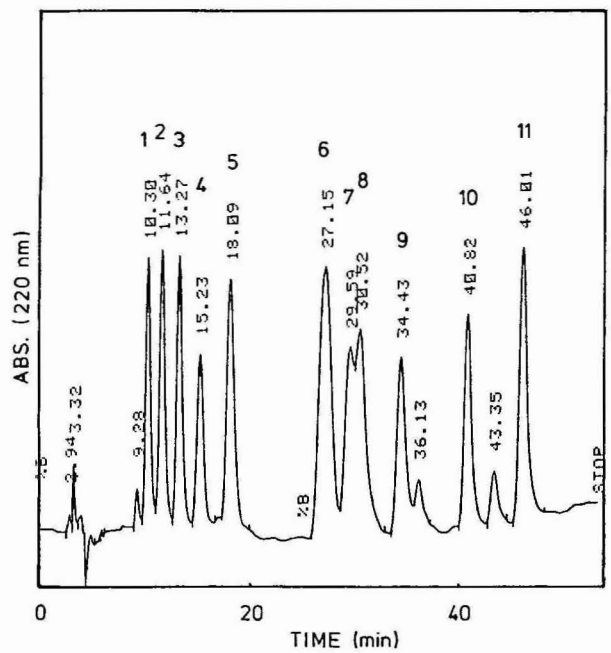

Fig. 12. Separation of angiotensins. Column details as in Fig. 6 except gradient elution starting from 24% B at a rate of 0.1% B/min for 25 min, then 0.5% B/min and column temperature: 26°C; amount of injection per peptide, approximately 1 μg (1 nmol based on A II). Peaks: 1, A III; 2, (Val[4])-A III; 3, A III inhibitor; 4, (Asn[1]–Val[5])-A II; 5, (Sar[1]–Ile[8])-A II; 6, (Sar[1]–Ala[8])-A II, (Sar[1]–Gly[8])-A II; 7, (Sar[1]–Thr[8])-A II; 8, (Sar[1]–Val[5]–Ala[8])-A II; 9, A II; 10, A I; 11, (Val[5])-A II.

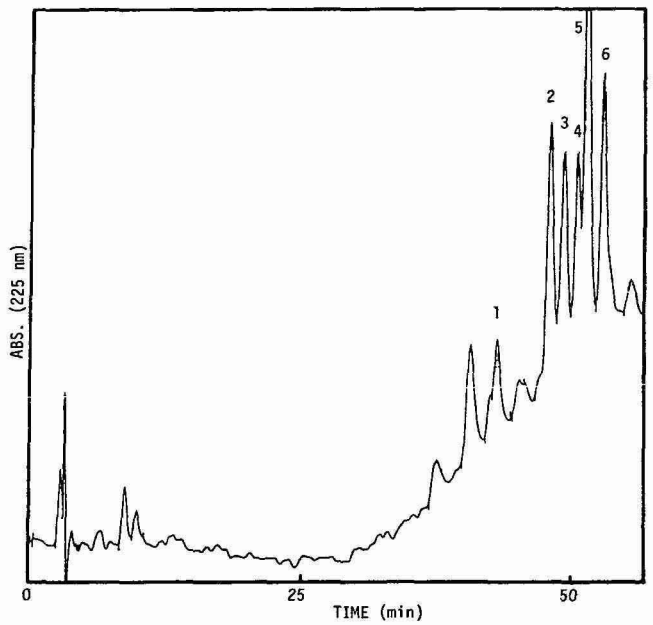

Fig. 13. Separation of some diastereomers of angiotensin I. Column details as in Fig. 6 except temperature: 50°C, and gradient program: linear starting from 24% B at a rate of 0.08% B/min for 25 min, then 0.25% B/min. Peaks: 1, (D-*allo*-Ile^5)–A I; 2, (D-Leu^{10})–A I; 3, (D-Phe^8)–A I; 4, (D-Pro^7)–A I; 5, A I; 6, (D-Asp^1–Ile^8)–A I.

4.3. Diastereomers of angiotensin I

A number of diastereomers of angiotensin I (AI) were separated by WAE-HPLC[19], as Fig. 13 illustrates. Peak identification is given in the legend of Fig. 13. In that work, separation of individual diastereomers of AI from their impurities was also demonstrated. As an example, separation of (D-Phe^8)-AI from its impurities is shown in Fig. 14. Peak 5 represents (D-Phe^8)-AI and other peaks corresponds to impurities characterized by fractional additions of combinations of Asp, Tyr or Arg, fractional deletions of His or reduced amounts of Ile, Val and Tyr (for more details see ref. 19).

4.4. Analogues and diastereomers of neurotensin

Neurotensin (NT) is a recently discovered peptide hormone which exhibits a broad spectrum of biological activity[20]. A large number of fragments, diastereomers and analogs of NT have recently been synthesized and tested for their biological activity[21].

Separation of a number of diastereomers and analogues of NT was achieved by WAE-HPLC[22], as shown in Figs. 15 and 16, respectively. Peak identification is given in the legend of the figures. Excellent recoveries between 90 and 98% of NT's tested were obtained[22]. Except for (D-Arg^9)-NT (peak 5 in Fig. 15), all diastereomers were completely separated from NT (peak 6 in Fig. 15). Also remarkable is the excellent resolution of (Phe^{11})-NT (peak 4) from (D-Phe^{11})-NT (peak 1). Five analogues of NT were also completely resolved from NT (peak 3 in Fig. 16).

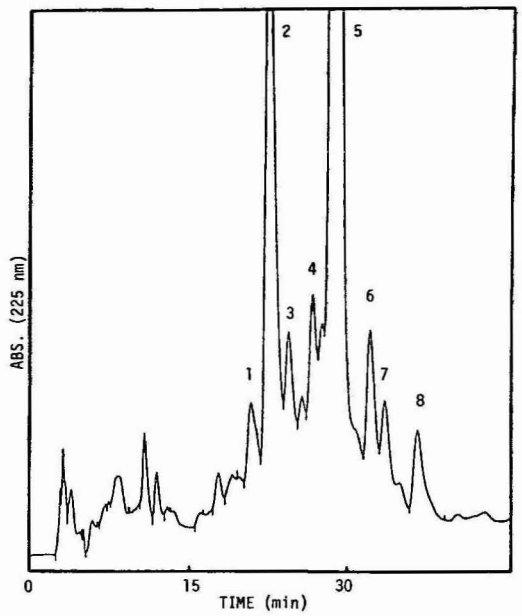

Fig. 14. Separation of (D-Phe[8])-A I from its impurities. Column details as in Fig. 6 except temperature: 30°C, and gradient program starting from 25% B at a rate of 0.5% B/min. Peak 5 represents (D-Phe[8])-A I. For other details, see ref. 19.

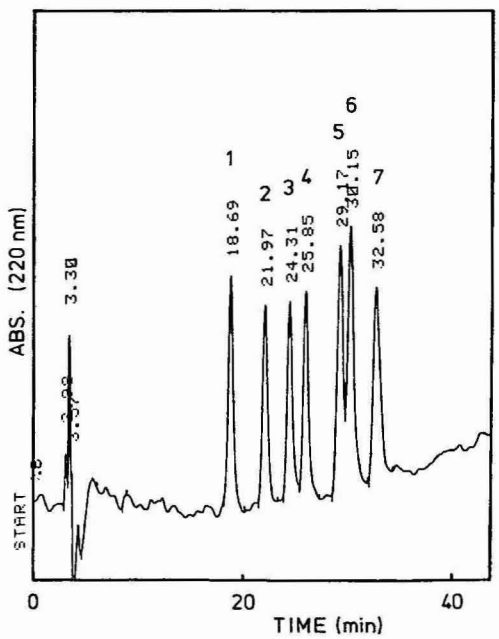

Fig. 15. Separation of some diastereomers of neurotensin. Column details as in Fig. 6 except temperature: 50°C, and gradient program: linear starting from 23% B at a rate of 0.3% B/min. Amount of injection per peptide, *ca.* 1 nmol. Peaks: 1, (D-Phe[11])-NT; 2, (D-Tyr[11])-NT; 3, (D-Pro[10])-NT; 4, (Phe[11])-NT; 5, (D-Arg[9])-NT; 6, NT; 7, (D-Glu[4])-NT.

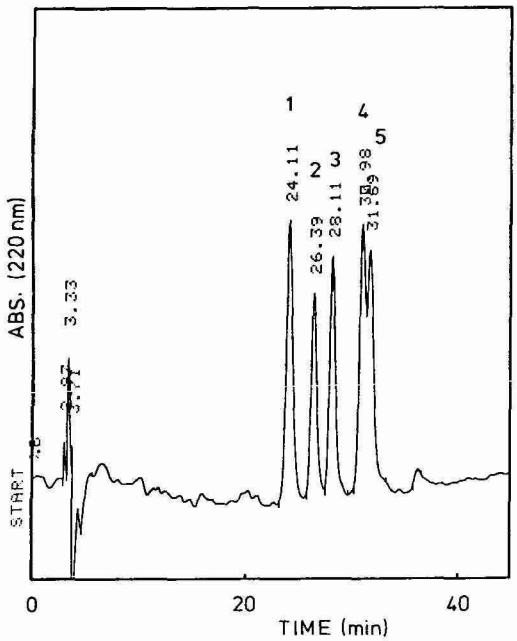

Fig. 16. Separation of some analogs of neurotensin. Column details as in Fig. 15 except temperature: 40°C. Peaks: 1, (Phe[11])–NT, (Trp[11])–NT; 2, (Leu[11])–NT; 3, NT; 4, (Lys[8])–NT; 5, (Lys[9])–NT.

5. EFFECT OF pH ON RETENTION

Separation of dipeptides by WAE-HPLC was carried out at pH 4.3 of the TEAA buffer[5]. For larger peptides, however, a pH value of 6.0 was found more suitable in terms of retention, peak symmetry and resolution[13]. The separation selectivity of the stationary phase used thus shows a quite strong dependence on the pH value of the buffer. For this reason, pH values between 4 and 6 should be explored to improve separation by WAE-HPLC of a given mixture of peptides.

The retention of acidic peptides depends even more strongly on the pH value of the eluent, as Fig. 17 clearly shows. A strong dependence of k' values of delta sleep-inducing peptide on pH above *ca.* 2.9 was observed. To keep a constant solvent strength, a 0.04 *M* formic acid solution was used and pH of the eluent was increased by adding triethylamine.

6. EFFECT OF TEMPERATURE

A strong effect of column temperature on retention and resolution of peptides separated by WAE-HPLC was observed. As an example, the dependence of retention times of angiotensins on temperature is given in Table 1 (ref. 18). An increase in column temperature from 26 to 50°C differently affected retention times of individual peptides. For instance, retention times of three A IIIs, (Asn[1]–Val[5])–A II, A II, A I, and (Val[5])–A II were increased by an increase in temperature. On the other hand, the increase of temperature caused a decrease in retention times of all five A's con-

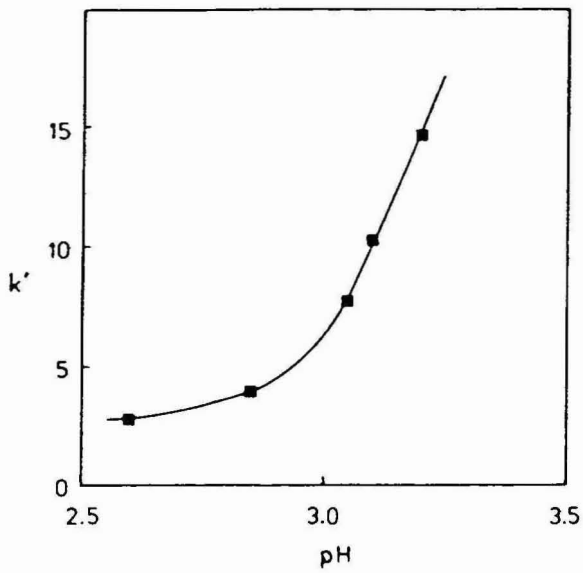

Fig. 17. Dependence of the k' value of DSIP (peptide No. 5 in Fig. 7) on the pH value of the eluent. Column as in Fig. 1. Eluent, 0.04 *M* formic acid, the pH value was adjusted by adding triethylamine. Flow-rate, 1.5 ml/min. Temperature, 60°C.

taining sarcosine. Consequently, resolution between the peptides of this particular mixture was greatly affected by a change in column temperature. For example, (Sar^1–Ala^8)-A II and (Sar^1–Gly^8)–A II coeluted at 26°C as Fig. 12 shows. But, they could be completely separated from each other at 50°C.

Retention times of analogs and diastereomers of NT were also significantly affected by column temperature as demonstrated in Table 2 (ref. 22). In this case, an

TABLE 1

DEPENDENCE OF RETENTION TIMES OF ANGIOTENSINS ON TEMPERATURE

Other column details as in Fig. 12.

Angiotensin	*Retention time (min) at*			
	26°C	*30°C*	*40°C*	*50°C*
A III	10.3	10.5	11.0	11.6
(Val^4)-A III	11.6	11.8	12.2	12.8
A III inhibitor	13.2	13.3	13.4	13.7
(Asn^1–Val^5)-A II	15.2	15.6	16.2	17.1
(Sar^1-Ile^8)-A II	18.1	17.8	17.1	17.1
(Sar^1-Ala^8)-A II	27.2	26.3	25.0	24.3
(Sar^1-Gly^8)-A II	27.2	26.3	26.2	26.8
(Sar^1-Thr^8)-A II	29.6	28.8	27.8	26.8
(Sar^1-Val^5-Ala^8)-A II	30.5	29.5	27.8	26.8
A II	34.4	35.5	38.3	40.8
A I	40.8	42.8	45.8	48.0
(Val^5)-A II	46.0	47.7	49.8	51.1

TABLE 2

DEPENDENCE OF RETENTION TIMES OF NEUROTENSINS ON TEMPERATURE

Other column details as in Fig. 15.

Neurotensin	*Retention time (min) at*		
	30°C	*40°C*	*50°C*
Fig. 15.			
(D-Phe^{11})-NT	16.1	17.5	18.7
(D-Tyr^{11})-NT	19.2	20.7	22.0
(D-Pro^{10})-NT	21.8	23.1	24.3
(Phe^{11})-NT	23.3	24.3	25.9
(D-Arg^{9})-NT	26.5	27.4	29.2
NT	27.6	28.2	30.2
(D-Glu^{4})-NT	29.9	31.2	32.6
Fig. 16.			
(Phe^{11})-NT	23.4	24.1	25.9
(Trp^{11})-NT	23.4	24.1	25.9
(Leu^{11})-NT	25.8	26.4	27.6
NT	27.6	28.1	30.0
(Lys^{8})-NT	30.3	31.0	32.8
(Lys^{9})-NT	30.9	31.7	33.4

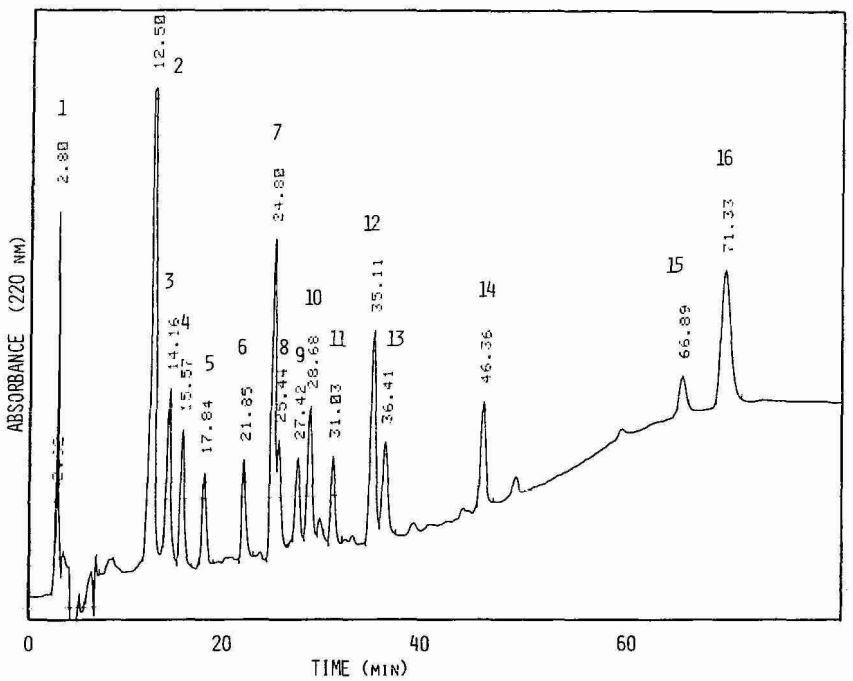

Fig. 18. Separation of a tryptic digest of RSMBP by WAE-HPLC. Column details as in Fig. 6 except temperature: 40°C, and gradient program: linear starting from 23% B at a rate of 0.7% B/min for 40 min, then 1% B. Peak identification and sequences are given in Table 3.

increase in temperature from 30 to 50°C caused an increase in retention times of all NT's tested.

7. COMPARISON OF RP- AND WAE-HPLC METHODS

RP- and WAE-HPLC methods for peptide separations were recently compared using a tryptic digest of rat small myelin basic protein (RSMBP)[23]. This small protein isolated from rat brain contains 127 amino acid residues[24] and expected to yield twenty-one peptide fragments and two arginine molecules upon digestion with trypsin. The tryptic digest of RSMBP was analyzed by WAE-HPLC using the conditions given in ref. 13 and by RP-HPLC using a solvent system containing 0.1% trifluoroacetic acid (TFA) in water and acetonitrile[25].

Fig. 18 shows the separation by WAE-HPLC, where a resolution of sixteen peaks was observed. Peak identification and sequences are given in Table 3, which indicates that the identified fragments cover the total sequence of RSMBP. Fig. 19 shows the separation by RP-HPLC of another aliquot of the same sample. The amino acid sequences of the peptides and their positions in the total sequence are given in Table 4. By deduction from the known sequence of RSMBP[24], the number of peptides accumulated near the void volume should total nine, because peaks 2–13 in Fig. 19 correspond to the remaining twelve tryptic peptides. The results showed that neither method resolved all of the peptides. All but two peptides were retained on the WAE column and several peaks contained two peptides, whereas some peptides had little or no retention on the RP column. Different selectivities of these two sep-

TABLE 3

PEAK IDENTIFICATION AND SEQUENCES IN FIG. 18

Peak	*Sequence*	*Position in sequence*
1	His–Arg	32, 33
	Gly–Arg	103, 104
	2 Arg	54, 127
2	Phe–Ser–Trp–Gly–Gly–Arg	111–116
3	His–Gly–Phe–Leu–Pro–Arg	26–31
4	Asn–Ile–Val–Thr–Pro–Arg	89–94
5	Gly–Leu–Ser–Leu–Ser–Arg	105–110
6	Ser–Gly–Ser–Pro–Met–Ala–Arg	120–126
7	Arg–Pro–Ser–Gln–Arg	5–9
	Thr–Thr–His–Tyr–Gly–Ser–Leu–Pro–Gln–Lys	64–73
8	Ser–Gln–Arg	74–76
9	Ac–Ala–Ser–Gln–Lys	1–4
	Gly–Ala–Pro–Lys	50–53
10	Thr–Pro–Pro–Pro–Ser–Gln–Gly–Lys	95–102
11	His–Gly–Ser–Lys	10–13
	Gly–Ser–Gly–Lys	55–58
12	Tyr–Leu–Ala–Thr–Ala–Ser–Thr–Met–Asp–His–Ala–Arg	14–25
13	Phe–Phe–Ser–Gly–Asp–Arg	44–49
	Asp–Ser–Arg	117–119
14	Asp–Ser–His–Thr–Arg	59–63
15	Asp–Thr–Gly–Ile–Leu–Asp–Ser–Ile–Gly–Arg	34–43
16	Thr–Gln–Asp–Glu–Asn–Pro–Val–Val–His–Phe–Phe–Lys	77–88

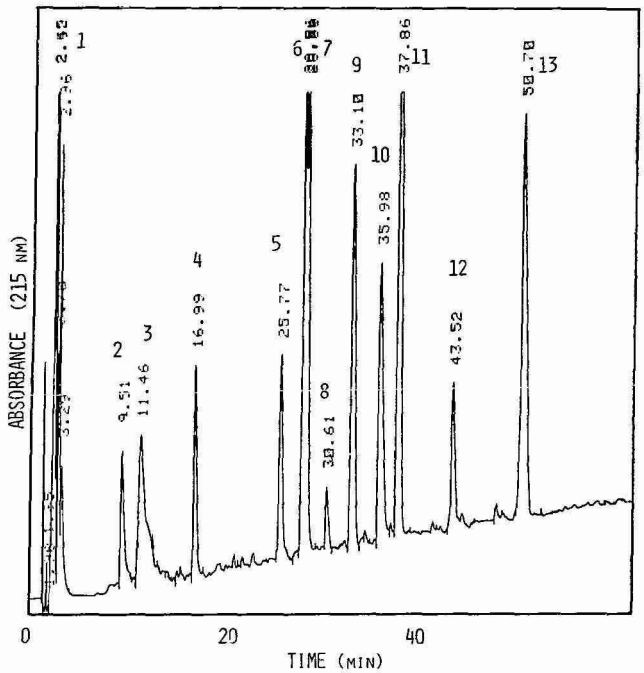

Fig. 19. Separation of a tryptic digest of RSMBP by RP-HPLC. Column: Supelcosil LC-8-DB (5 μm), 15 × 0.46 cm. Temperature: 30°C. Eluents: A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile. Gradient program: linear starting from 0% B at a rate of 0.5% B/min. Flow-rate: 1.2 ml/min. Peak identification and sequences are given in Table 4.

aration principles were demonstrated by collecting the peptides near the void column of the RP column (designated as peak 1 in Fig. 19) and injecting them onto the WAE column. A resolution of six peaks corresponding to nine peptides and two arginine's was observed[23]. Except for the two dipeptides, all peptide fragments were retained on the WAE column in contrast to the RP column, and three of them could be obtained in pure form (for details, see ref. 23). In another instance, two peptides, which were slightly resolved on the RP-column (peaks 6 and 7 in Fig. 19), were collected and injected onto the WAE column. Fig. 20 shows the complete separation of these peptides on the WAE column. These examples excellently demonstrate the combined use of RP- and AE-HPLC methods for isolation of peptides, which were not resolved by using only one method. It is important to note that the RP-HPLC has not been exhaustively studied by using solvent systems other than the TFA system and the total capability of this technique is not judged here.

In some other instances, as might be expected, RP-HPLC provided better resolution of certain peptides than did the WAE-HPLC methods (for more details, see ref. 23). The results obtained in that work strongly suggested that the combined use of these two methods of separation, which utilize different selectivities, can provide even more resolving power and thus yield a high probability for the complete separation of a given mixture of peptides into its components.

The combined use of RP- and WAE-HPLC was also demonstrated by Lemke

TABLE 4

PEAK IDENTIFICATION AND SEQUENCES IN FIG. 19

Peak	*Sequence*	*Position in sequence*
1	Ac–Ala–Ser–Gly–Lys	1–4
	His–Gly–Ser–Lys	10–13
	His–Arg	32–33
	Gly–Ala–Pro–Lys	50–53
	2 Arg	54, 127
	Gly–Ser–Gly–Lys	55–58
	Asp–Ser–His–Thr–Arg	59–63
	Ser–Gln–Arg	74–76
	Gly–Arg	103–104
	Asp–Ser–Arg	117–119
2	Arg–Pro–Ser–Gln–Arg	5–9
3	Thr–Pro–Pro–Pro–Ser–Gln–Gly–Lys	95–102
4	Ser–Gly–Ser–Pro–Met–Ala–Arg	102–126
5	Asn–Ile–Val–Thr–Pro–Arg	89–94
6	Thr–Thr–His–Tyr–Gly–Ser–Leu–Pro–Gln–Lys	64–73
7	Phe–Phe–Ser–Gly–Asp–Arg	44–49
8	Gly–Leu–Ser–Leu–Ser–Arg	105–110
9	Tyr–Leu–Ala–Thr–Ala–Ser–Thr–Met–Asp–His–Ala–Arg	14–25
10	His–Gly–Phe–Leu–Pro–Arg	26–31
11	Phe–Ser–Trp–Gly–Gly–Arg	111–116
12	Asp–Thr–Gly–Ile–Leu–Asp–Ser–Ile–Gly–Arg	34–43
13	Thr–Gln–Asp–Glu–Asn–Pro–Val–Val–His–Phe–Phe–Lys	77–88

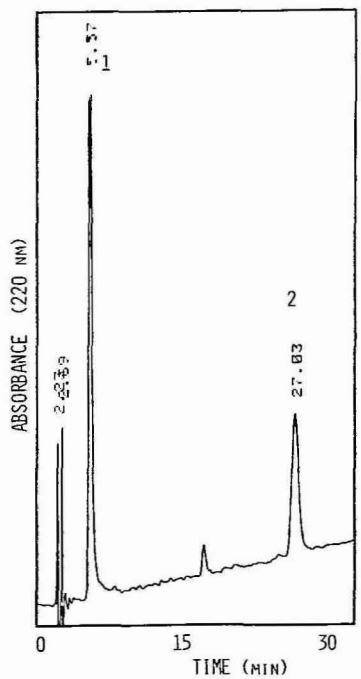

Fig. 20. Separation of peptides eluted from RP column (peaks 6 and 7 in Fig. 19) by WAE-HPLC. Column details as in Fig. 18 except for gradient program: starting from 45% B at a rate of 0.5% B/min. Peaks 1 and 2 correspond to peaks 6 and 7 in Fig. 19, respectively.

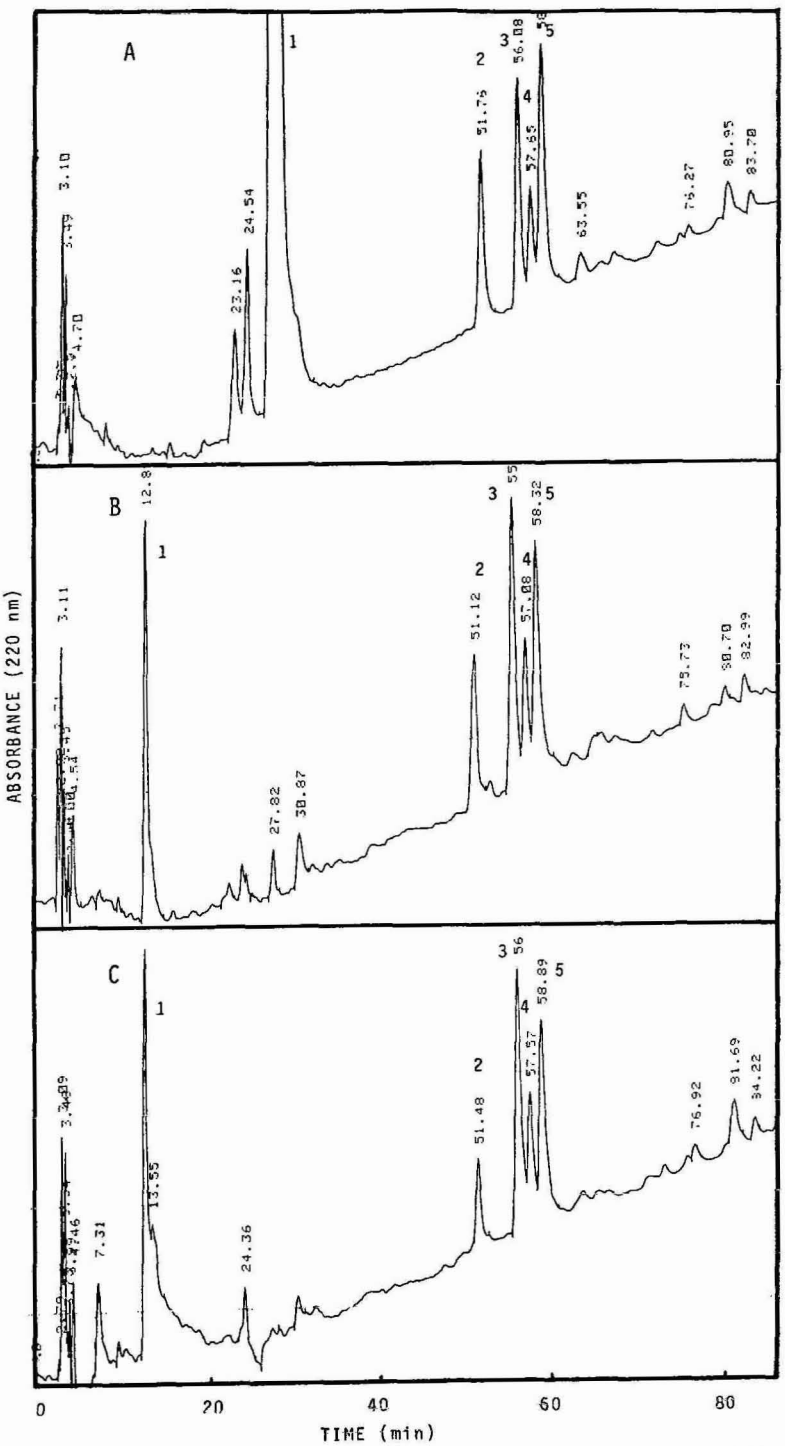

Fig. 21. (A) Separation of γ-irradiated L-Ala–L-Ala by WAE-HPLC. Column, MicroPak AX-5 (5 μm), 30 × 0.4 cm; temperature, 40°C; eluent and flow as in Fig. 6; gradient elution starting from 25% B with a rate of 0.75% B/min. (B) and (C) γ-irradiated L-Ala–L-Ala after hydrolysis with aminopeptidase M and leucine aminopeptidase, respectively.

et al.[26]. Separation of all peptides contained in a subtilisin digest of purple membrane protein bacteriorhodopsin was achieved when peptide fragments derived from a RP-column were injected onto a WAE-column under the conditions given in ref. 5.

Furthermore, Margolis and Dizdaroglu (see above and ref. 19) compared the results of separation of AI diastereomers by WAE-HPLC with those obtained by RP-HPLC[27]. This comparison also strongly suggested[19] that these two HPLC methods of different principles are complementary and their combined use can confidently assess the purity of a given peptide preparation.

8. SEPARATION OF RADIATION-INDUCED PRODUCTS OF PEPTIDES

The WAE-HPLC method was recently applied to separation and isolation of radiation-induced products of aliphatic peptides[28]. Digestibility by exopeptidases of

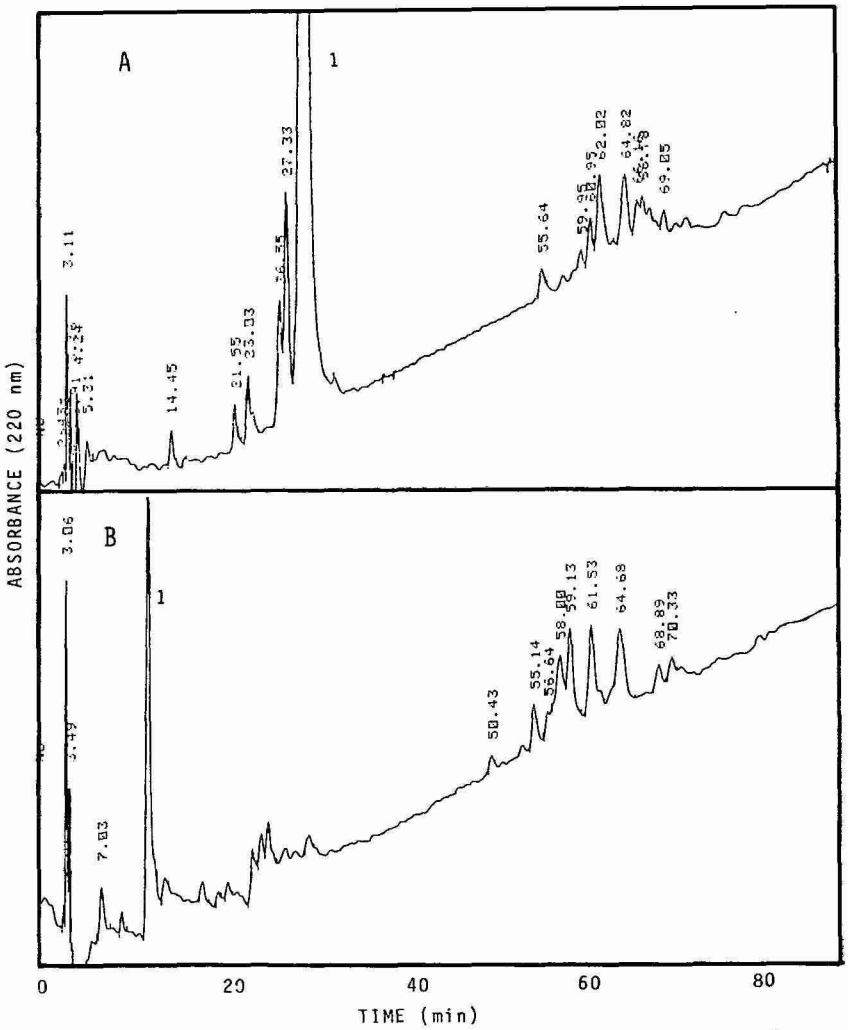

Fig. 22. (A) Separation of γ-irradiated tetra-L-Ala by WAE-HPLC. Column details as in Fig. 21. (B) γ-irradiated tetra-L-Ala after hydrolysis with aminopeptidase M.

peptides cross-linked by ionizing radiation was also investigated by using the same method[29]. Fig. 21A shows the separation of γ-irradiated L-Ala–L-Ala. Peak 1 represents this dipeptide and peaks 2–5 correspond to products of radiation-induced crosslinks, which were identified subsequently by gas chromatography–mass spectrometry[28]. Figs. 21B and C show the elution profiles of γ-irradiated L-Ala–L-Ala after digestion with aminopeptidase M and leucine aminopeptidase, respectively. Hydrolysis with two carboxypeptidases yielded similar chromatograms (not shown here). These results clearly indicate the non-digestibility of the crosslinked products under the conditions which hydrolyzed completely L-Ala–L-Ala. WAE-HPLC of γ-irradiated L-Ala–L-Ala–L-Ala–L-Ala (tetra-L-Ala) also showed the presence of radiation-crosslinked products (Fig. 22A)[28]. Partial digestion of these products by amino- and carboxypeptidases was obtained as revealed by their HPLC analysis under the same conditions[29]. As an example, Fig. 22B shows the HPLC profile of γ-irradiated tetra-L-Ala after digestion with aminopeptidase M.

While WAE-HPLC was the method of choice for the analysis of γ-irradiated aliphatic peptides, RP-HPLC yielded better results in analysis of γ-irradiated aromatic peptides[30] and was also successfully used for investigation of their enzymatic digestibility[29].

9. CONCLUSIONS

This survey shows that the WAE-HPLC method recently developed is an excellent approach for peptide separations. A broad range of peptides including diastereomeric and other closely related peptides can be chromatographed and successfully separated. The buffer used allows detection of peptides at wavelengths in the range 210–225 nm. Its volatility facilitates a convenient and efficient recovery of salt free peptides for further use and study. The column used provides high recoveries of chromatographed peptides. Because of these features, the WAE-HPLC method could be quite useful for peptide separation and identification in the rapidly developing field of HPLC–mass spectrometry[31,32].

Furthermore, this review indicates that, in some instances, RP- and WAE-HPLC are complementary. This means that their combined use can provide the optimal separation of a given peptide mixture into its components or assess the purity of a peptide preparation.

10. ACKNOWLEDGEMENTS

The author acknowledges the contributions of Dr. H. C. Krutzsch (National Institutes of Health), Dr. M. G. Simic, Dr. S. A. Margolis (both National Bureau of Standards), Dr. S. St-Pierre and Dr. F. Rioux (both University of Sherbrooke, Canada) to the papers reviewed here.

11. SUMMARY

In this survey, the principles and applications of a method recently developed for peptide separations are given. This method uses a bonded weak anion-exchange column and mixtures of volatile triethylammonium acetate buffer and acetonitrile as

eluent. Its applications to the separation of a large number of peptides including diastereomeric and other closely related peptides are discussed. Separation of the enzymatic digests of some proteins is also presented. The complementary use of this method to the reversed-phase methods is outlined and their combined use for separation of enzymatic digests of proteins and assessment of purity of synthesized peptides is demonstrated. The results reviewed show that the weak anion-exchange method is an excellent approach for peptide separations and could be an important partner of reversed-phase methods for achieving optimal results.

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CHREV. 191

ISOELECTRIC FOCUSING IN NON-AMPHOTERIC BUFFERS

CATASTROPHE AND NON-CATASTROPHE THEORIES

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CONTENTS

1. INTRODUCTION

Modern isoelectric focusing (IEF) is still based on the rigorous theoretical treatment of Svensson-Rilbe[1,2] who more than 20 years ago, derived the fundamental equations governing the system at the steady state and defined the minimum requirements for correct functioning of the technique. Central to his theory is the concept of "carrier ampholytes": the buffers used in IEF have to be amphoteric, so that they also would seek a stationary position in the system; moreover, they have to be "carriers". This is a more subtle concept, but just as fundamental: they have to be "carriers" of buffering power, so that they would prevent local pH changes, and of conductivity, so as to ensure an unhindered flow of current through the focusing cell. Over about 25 years, the technique has proved very suitable for this job[3].

Is it possible, or is there a need, to break away from such a well constructed and much explored methodology? A need definitely arose, as conventional IEF had begun to show signs of age, such as: (a) cathodic drift; (b) lack of even conductivity and buffering capacity; (c) extremely low and unknown ionic strength (I) and (d) limited load capacity, mostly due to isoelectric precipitation caused by the low I environment. As for the possibility, this is a complex story: over the years, several groups have tried to break away from Svensson's theory, by resorting to different approaches of what has been generally termed "non-amphoteric buffer isoelectric focusing" (NAB-IEF) (let us recall here that, around 1955, this concept had been amply described by Kolin[4] with his "artificial" pH gradients).

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2. THEORY AND RESULTS

2.1. Chrambach's theory

According to this theory, IEF can be viewed as a particular form of isotachophoresis (ITP), where a separation within a mobile pH gradient, as in ITP, is replaced by a separation within a stationary pH gradient, typical of IEF (for a review, see, ref. 5; this concept had been proposed long ago by Routs[6]). While this can be easily accepted for conventional IEF in amphoteric buffers, it is not immediately apparent for NAB-IEF: how can non-amphoteric buffers form a stationary (and also a "natural") pH gradient? They have to be arrested, and this blocked "stack" or "train" of buffers is obtained by a neutralization process, *i.e.*, a protonation with acidic species and a deprotonation mechanism with basic compounds. There is a simple way of achieving this: free acids and free bases are deprived of counter ions (*i.e.*, of titrants) and are allowed to migrate into a pH region where they will have only the hydrolytic products of water as counter ions. As an example, we report in Table 1 and Fig. 1 the data of Chrambach and Hjelmeland[5] on the generation of a "stationary" and "natural" pH gradient between pH 10.29 and 12.18 solely with the use of six free bases, with pK values ranging from 6.88 to 10.35. While we agree that such a system can generate a pH gradient, as long ago demonstrated by Schumacher[7] and subsequently by Pettersson[8] and Stenman and Grasbeck[9] for free acids, we hesitate to call it "stationary", a fundamental prerequisite of true IEF. We could give a number of reasons, but there is some obvious macroscopic, negative evidence: how

TABLE 1

PREDICTED PROPERTIES OF AN ISOELECTRIC FOCUSING SYSTEM COMPOSED OF SIX NON-AMPHOTERIC BASES

From Chrambach and Hjelmeland[5]. r = Ionic mobility relative to Na^+; φ = flux (mol cm^{-2} sec^{-1}); $\bar{r}$ = relative net mobility; κ = specific conductance (10^{-6} Ω^{-1} cm^{-1}); v = boundary displacement rate (cm^3 C^{-1}); v = boundary velocity (cm per day).

Parameter	*Ethanolamine*	*Morpholine*	*N-Ethyl-morpholine*	*N-(2-Hydroxy-ethyl)-morpholine*	*Lutidine*	*Bis–Tris*
r*	0.86	0.73	0.62	0.61	0.60	0.38
pK_a*	10.35	8.85	8.03	7.19	7.00	6.88
Concentration (M)	1.00	0.87	0.76	0.75	0.74	0.49
pH	12.18	11.40	10.96	10.53	10.43	10.29
φ	$1.50 \cdot 10^{-2}$	$2.85 \cdot 10^{-3}$	$1.19 \cdot 10^{-3}$	$4.55 \cdot 10^{-4}$	$3.68 \cdot 10^{-4}$	$3.93 \cdot 10^{-4}$
$\bar{r}$	$1.29 \cdot 10^{-2}$	$2.08 \cdot 10^{-3}$	$7.37 \cdot 10^{-4}$	$2.78 \cdot 10^{-4}$	$2.21 \cdot 10^{-4}$	$1.45 \cdot 10^{-4}$
κ	$1.91 \cdot 10^{-3}$	$3.08 \cdot 10^{-4}$	$1.09 \cdot 10^{-4}$	$4.11 \cdot 10^{-5}$	$3.27 \cdot 10^{-5}$	$2.21 \cdot 10^{-5}$
v	$1.85 \cdot 10^{-3}$	$1.85 \cdot 10^{-3}$	$1.85 \cdot 10^{-3}$	$1.85 \cdot 10^{-3}$	$1.85 \cdot 10^{-3}$	$1.85 \cdot 10^{-3}$
v**	$6.85 \cdot 10^{-6}$	$6.85 \cdot 10^{6}$	$6.85 \cdot 10^{-6}$	$6.85 \cdot 10^{-6}$	$6.85 \cdot 10^{-6}$	$6.85 \cdot 10^{-6}$
w (cm)	$9.33 \cdot 10^{-3}$	$4.29 \cdot 10^{-3}$	$1.67 \cdot 10^{-3}$	$4.08 \cdot 10^{-3}$	$1.74 \cdot 10^{-3}$	

* Values at 0°C.

** 1 mA per 0.27 cm^2 of gel.

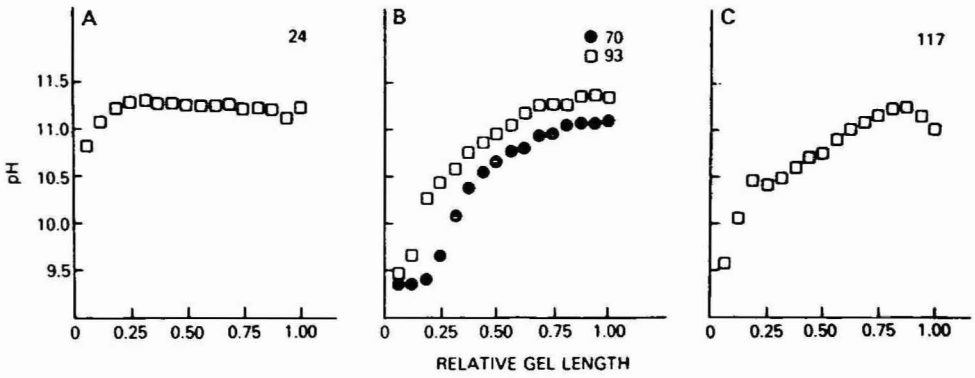

Fig. 1. Experimental pH gradient formed by the moving boundary system of six non-amphoteric bases having p*K*s and molarities as defined in Table 1. A, measured after 24 h of electrophoresis; B, after (●) 70 and (□) 93 h; C, after 117 h. (From Chrambach and Hjelmeland[5]).

can a buffer possibly have any buffering capacity (and also contribute to the conductivity) when it is confined to a pH region more than 3 pH units removed from its p*K* (and on its deprotonated side also)? We therefore re-simulated their computer simulations with the program we have developed for a very peculiar kind of NAB-IEF, namely immobilized pH gradients(IPG)[10–12], in order to demonstrate how to use correctly these buffers for IEF. Our computer was required to simulate and optimize what we believe are the fundamental physico-chemical parameters of an IEF separation: the slope of the pH gradient (and its optimization in terms of minimal deviation from linearity) and accompanying buffering power (β) and ionic strength (I). When we took the six buffers in Table 1, with their relative molarities, and asked the computer to calculate the β and I values along the pH 10.29–12.18 potential gradient, it could not do so, since in our computer program both a buffer and a titrant are required. We had to calculate these values manually, by assuming that the six bases are sequentially distributed along the pH gradient, starting with Bis–Tris at pH 10.29 and ending with ethanolamine at pH 12.18 and occupying "boxes" with lengths proportional to their respective absolute amounts present in the system. The

TABLE 2

BUFFERING POWER (β) AND IONIC STRENGTH (I) OF CHRAMBACH'S ARRESTED STACK OF FREE BASES

Parameter	*pK*					
	6.88	*7.00*	*7.19*	*8.03*	*8.85*	*10.35*
Concentration (m*M*)	490	740	750	760	870	1000
pH_m*	10.4	1.65	10.95	11.25	11.6	12.0
I (equiv. l^{-1})	0.148	0.166	0.130	0.458	1.544	21.9
β (equiv. l^{-1} pH^{-1})	0.34	0.38	0.30	1.05	3.55	49.3

* pH at mid-point of each "isotachophoretic" zone, having a length proportional to the total amount present in the system.

results are reported in Table 2. It can be seen that the β and I values are much too low to ensure any buffering power and conductivity for the system, and as such this cannot be included among the natural pH gradients characteristic of true IEF, where the concept of "carrier" buffers is indissolubly linked to the generation of a pH gradient (in IPGs, we give as acceptable average values β = 3–4 mequiv. $pH^{-1}\ l^{-1}$

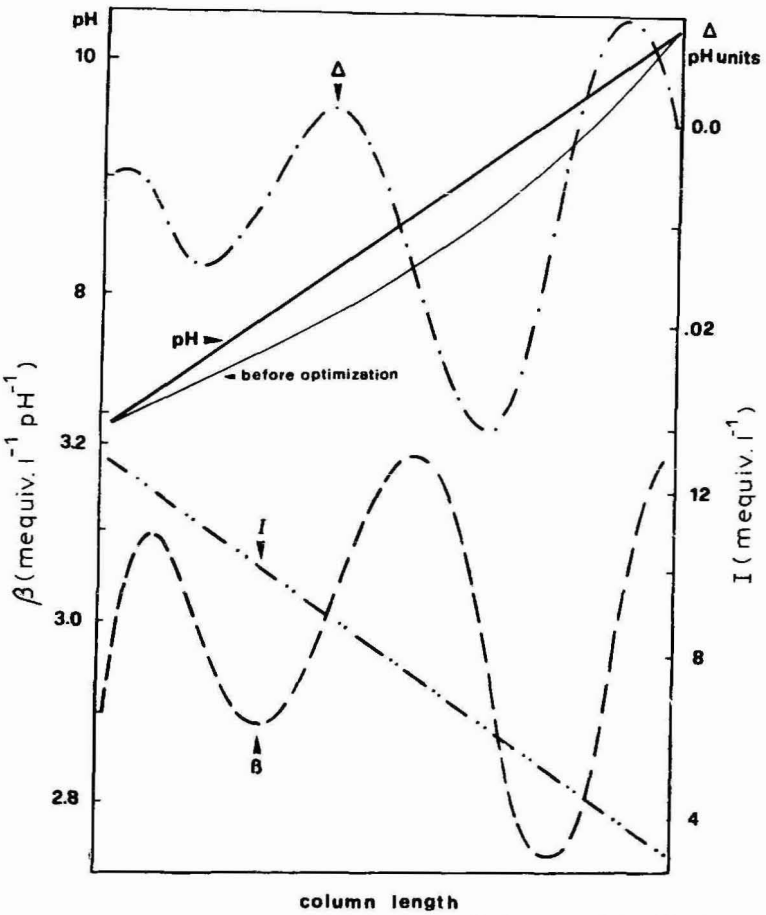

Fig. 2. pH gradient, deviation from linearity (Δ), buffering power (β) and ionic strength (I) of Chrambach's system (Fig. 1) when titrated between pK (min) (pH 6.88) and pK(max) (pH 10.35). It has been assumed that the same system of six non-amphoteric bases as in Fig. 1 are Immobilines and they have been titrated in the correct pH range with an acidic counter ion (Immobiline of pK 0.8). If the six bases are used in the same molarity ratios as in Fig. 1, a bow-shaped pH gradient is obtained ("before optimization" line). When the molarity ratios of the six buffers are changed according to the optimization algorithm of ref. 12, the upper, linear pH gradient is generated. The Δ, β and I values refer to the "optimized system". The concentrations of each base were as follows: pK 6.88, 2.09 mM; pK 7.00, 1.66 mM; pK 7.19, 0.80 mM; pK 8.03, 1.85 mM; pK 8.85, 3.82 mM; pK 10.35, 5.00 mM. For the titrant (Immmobiline of pK 0.8): 13 mM in the acidic chamber and 2.6 mM in the basic reservoir. These concentrations are adjusted so as to give an average buffering power of 3 mequiv. l^{-1} pH^{-1} (note that in the original system in Fig. 1 the molarities of the buffering ions are 200–500-fold higher, ranging from 490 to 1000 mM!). This "immobilized pH gradient" was generated with the aid of a two-vessel gradient mixer, according to the "same concentration" formulation, as explained in ref. 11.

and I = 4–5 mequiv. l^{-1}). Certainly such β and I values could be obtained even in this system of six bases, but by utilizing enormous molarity values (> 10 M), definitely incompatible with protein integrity and with an electrophoretic process. That this system cannot be stationary as well is amply demonstrated by the data of the authors who claim it to be so (see Fig. 1C). There is another reason why these gradients will not have any focusing action on proteins: the conductivity from pH 10.29 to 12.18 will be dictated primarily by the free OH^- in solutions (and the equivalent amount of protonated base), which means that it will increase substantially towards the cathode, leaving behind a high field strength at the anodic side. Over much of the pH gradient (above pH 11) there will be too little voltage drop to ensure any focusing power.

However, if we now assume these buffers to be Immobilines, and we titrate them with an acidic counter ion between pK (min) (pH 6.88) and pK (max) (pH 10.35), the situation is definitely brighter: it is possible to generate a decent pH course with acceptable β and I values (Fig. 2, thin solid line). Even in the correct pH range, however, the system does not behave well when using for the simulation the concentrations of the various buffers given in Table 1. After extensive modification of the relative ratios by our optimization algorithm, a very smooth pH course may be generated (Fig. 2, thick solid line).

2.2. "Chemically" immobilized pH gradients

There is not much to be said here, except that the system works, it allows full control of the experimental parameters (pH gradient width and slope, β and I courses)[10,12], complete choice of any pH gradient (from ultra-narrow ranges, *e.g.*, 0.01 pH unit/cm separation distance up to the widest possible span, pH 3–10) and, of course, generates indefinitely stable pH gradients. IPGs are, in a way, a particular case of NAB-IEF: as non-amphoteric buffer focusing, in reality, would never work, the problem has been solved by resorting to non-amphoteric, but bifunctional buffers. These chemicals are acrylamide derivatives, with pK values spaced at regular intervals along the pH scale [pK (min) = 3.6, pK (max) = 9.3] and they are utilized according to "canonical" principles (the "carrier" concept of Svensson!), *i.e.*, for generating pH gradients by titration with a counter ion around their respective pK values (ideally 0.5 pH unit above and 0.5 below the pK) where they exhibit maximum buffering power and substantial conductivity. The gel slab is cast with the aid of a gradient mixer, the two vessels being titrated one to pH (min) (*e.g.*, in the dense solution) and the other to pH (max) (*e.g.*, in the light solution). Therefore, when the gel cassette is filled up, it already contains a linear pH gradient, existing prior to the electrophoretic process itself: this system appears to have all the prerequisites of the "artificial" pH gradients according to Kolin[4]. However, a moment later, as the monomers polymerize to form the gel network, the non-amphoteric buffers are covalently linked to the matrix, and therefore the entire pH gradient is insolubilized (copolymerized, grafted, immobilized, etc.); this system is indefinitely stable to the flow of electric current and thus now exhibits all the prerequisites of the natural pH gradients according to Svensson[1,2]. Thus, by burning at the stake Kolin and Svensson, from their ashes this new "arab phoenix" was born, with the virtues of both and none of their defects. That IPGs are the new highway (or star port) to the year 2000 is already amply documented[13].

2.3. *"Physically" immobilized pH gradients*

Yet another approach to NAB-IEF comes from Bier's group, who has extensively developed highly sophisticated theories and models over the years. Their results were summarized in a recent paper by Bier *et al.*[14] and are exemplified in Fig. 3, taken from the same paper. They were finally able to generate "quite stable pH gradients formed using a simple system of a weak acid and a weak base (around neutrality), mixed in the proportion required to cover the desired pH range". In the simple case of Fig. 3, a gradient of a buffering acid (cacodylate, pK 6.2), varying linearly from 4 to 2 mM (anode to cathode), is titrated with a reciprocal linear gradient of buffering base (Tris, pK 8.3) ranging from 2 to 4 mM (anode to cathode). Bier *et al.* stated that their system defies ready classification in terms of conventional modes of electrophoresis as it is not isoelectric focusing, because none of the components of the buffer is isoelectric, and it is not IPG, because their buffers are not immobilized. However, close scrutiny of their data reveals that they have some of both attributes. The similarity with IPGs is striking. An analogous situation occurs in IPGs in the pH region 4.4–6.2, with Immobilines of pK 4.4 (a weak acid) and pK 6.2 (a weak base), where the system is used under conditions such that the two components act simultaneously as a buffer and as a titrant. As can be seen in Fig. 3A, the concentrations of the two are reciprocal, symmetrical linear gradients, generating a pH gradient from 6.21 (the pK of cacodylate, because here the [cacodylate]/[Tris] ratio is 2:1) to pH 8.3 (the pK of Tris, because here the [Tris]/[cacodylate] is 2:1). However, the pH gradient is not linear, but slightly sigmoidal, because ΔpK = 2.09, as predicted by our computer modelling[11]. Such a situation was indeed fully predicted by our general theory on IPGs[15]. We have in fact re-simulated their data with our computer algorithm, by assuming the two species to be Immobilines. As shown in Fig. 4, we in fact obtain the same results: the expected pH gradient is identical in the two instances, except that the deviation from linearity is high (the

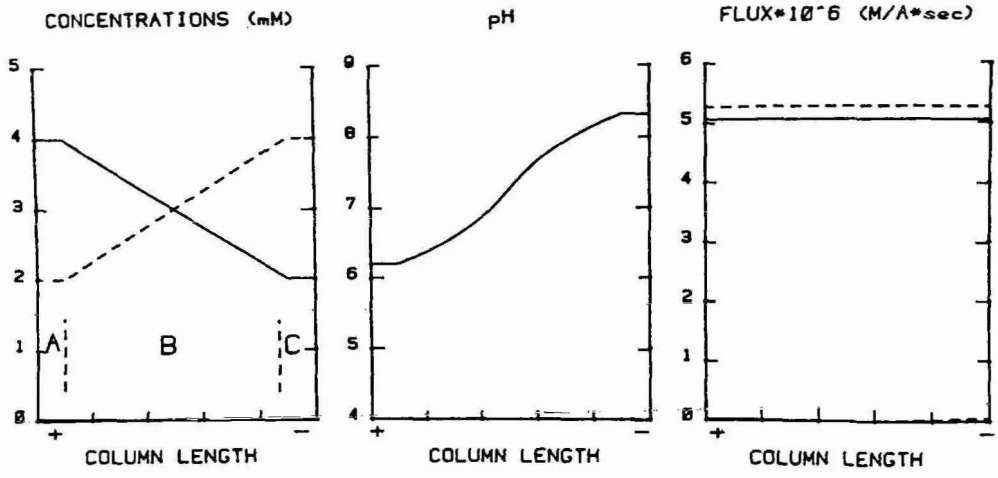

Fig. 3. Schematic representation of the courses of the concentration of Tris-cacodylate and their respective fluxes and of the resulting pH gradient. Microcomputer-calculated flux values are also plotted, assuming for Tris (dashed lines) a mobility of 2.42 $cm^2\ V^{-1}\ sec^{-1}$ and a pK of 8.3, and for cacodylate (solid lines) a mobility of 2.31 $cm^2\ V^{-1}\ sec^{-1}$ and a pK of 6.21. (From Bier *et al.*[14].)

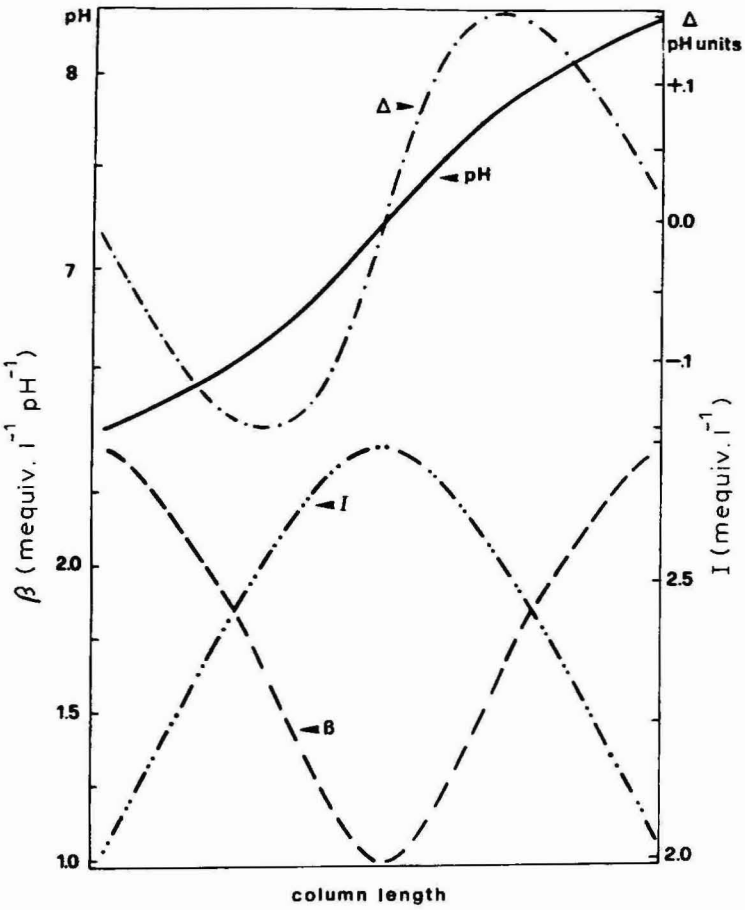

Fig. 4. pH gradient, deviation from linearity (Δ), buffering power (β) and ionic strength (I) of Bier *et al.*'s system in Fig. 3. Their data were re-calculated by using the same molarity and pK values and the same pH interval as in Fig. 3, except that Tris and cacodylate were assumed to be two Immobilines (*i.e.*, with mobility = 0, flux = 0, diffusion coefficient = 0). Note that the shapes of the expected pH gradient are identical in the two different computer models. Our computer simulation predicts that Bier *et al.*'s gradient is fully compatible with a well functioning IEF system (ideally, however, we prefer to have an average β = 3 mequiv. l^{-1} pH^{-1}, so that the concentrations of the two species should be approximately doubled).

maximum excursion, positive + negative, is 0.3 pH unit, *i.e.*, 15% of the stated pH interval; for typical Immobiline gradients, both with narrow and with extended ranges, the deviation is contained within less than 1% of the generated pH span). We could also simulate the β and I courses (Fig. 4, lower part); as expected, they appear as two reciprocating, bell-shaped functions, with a minimum of β power half-way between the two peaks, corresponding to a maximum of ionic strength (because, by titrating the two species "inside" the two pKs, we ensure conditions of maximum ionization of the buffering groups). While, with true Immobilines, we can arrange for smoother β and I courses, the physico-chemical parameters of Bier *et al.*'s system are acceptable and compatible with a well functioning IEF set-up. While it is true that the buffers here are not "chemically immobilized", they nevertheless

ensure substantial stability of the system because they are immobilized by physical laws. The fundamental requirement of Bier *et al.*'s gradients is that the flux of the two species is constant across the whole length of the column. In fact, they introduced a parameter ρ, which predicts the stability of the system, defined as

$$\rho = \frac{M_a^{A-} M_b^{C+}}{M_b^{A+} M_a^{C-}}$$

where M is the concentration of the acid (a^-) and base (b^+) in reservoirs A and C. Only when $\rho = 1$ would we have perfect migrational stability, and this is one of the weaknesses of the system: as shown in Fig. 3, the flux of Tris is slightly higher than the flux of cacodylate, and eventually the system is bound to decay.

2.4. Steady-state rheoelectrolysis

Another approach to the generation of useful and stable pH gradients from simple, two-component, non-amphoteric buffers was proposed in 1978 by Rilbe[16]. The idea is that it should be possible to create a stable pH course in a suitable electrolyser by balancing the internal electrical and diffusional mass flows by external mass flows generated by the pumping of anolyte to the catholyte and *vice versa* (hence the term rheoelectrolysis, *i.e.*, external hydrodynamic flow coupled to internal electrophoretic transport). In this system the steady state would generate a useful pH gradient provided the following criteria were fulfilled: (i) the transference numbers and the diffusion coefficients of the buffer ions are constant in the pH interval chosen; (ii) the compositions of the buffer system at the ends of the electrolyser are kept constant with time; (iii) no net liquid flows through the electrolyser are present; and (iv) losses of buffer constituents due to anodic oxidation, cathodic reduction, evaporation or precipitation are negligible. The system was further developed by Jonsson and Fredriksson[17] and the theory expanded by Rilbe[18–20].

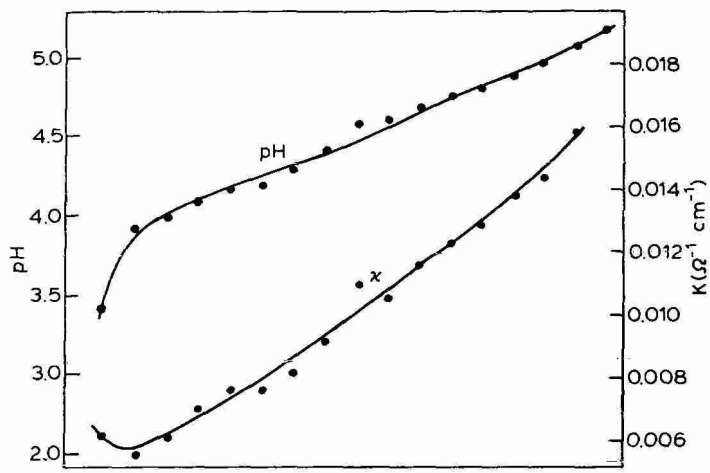

Fig. 5. Steady-state rheoelectrolysis of an acetate buffer solution. Experimental pH and conductivity courses in the electrophoretic cell at the steady state. (From Rilbe[15].)

Fig. 5 shows one of Rilbe's experiments for generating a linear, pH 3.9–5.2 course by steady-state rheoelectrolysis of an acetate buffer solution [three cases have been considered: (a) a buffer composed of a weak acid and its salt with a strong base (as shown in Fig. 5); (b) a buffer composed of a weak base and its salt with a strong acid; and (c) a buffer containing a salt of a weak acid and a weak base]. By the same reasoning, assuming acetate to be an Immobiline with pK 4.6, we re-simulated Rilbe's data in our computer system. As shown in Fig. 6, a smooth pH gradient is obtained in the pH 3.9–5.2 region with a maximum deviation from linearity of about 6% of the generated pH interval and with substantial average β and I values, compatible with a well performing IEF system (note that the slopes of Rilbe's conductivity profile in Fig. 5 and of our ionic strength curve in Fig. 6 are very similar, as the quantities are interchangeable).

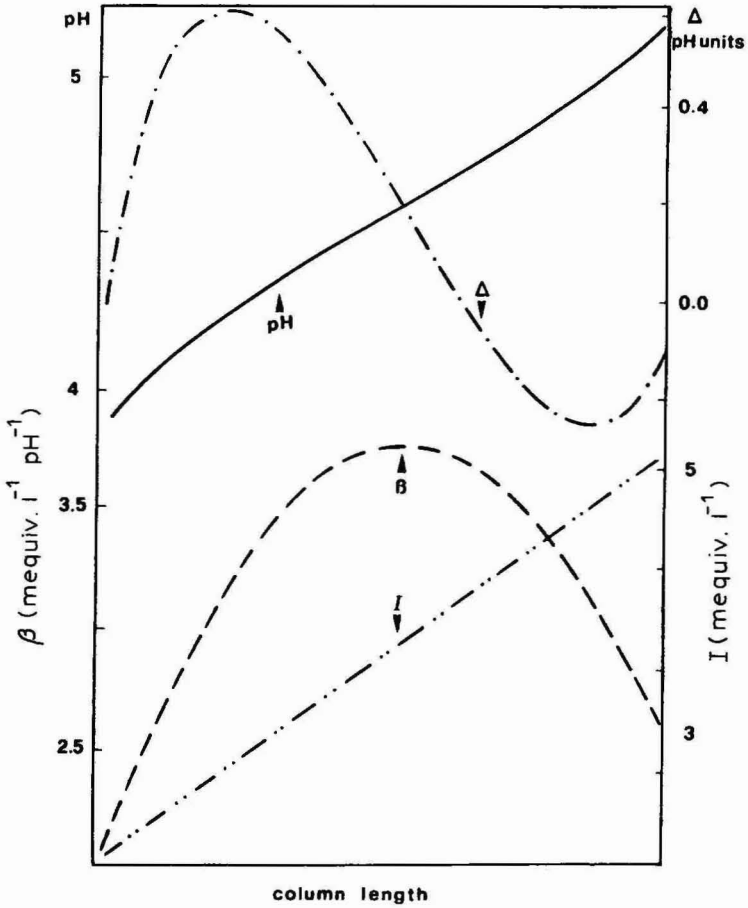

Fig. 6. pH gradient, deviation from linearity (Δ), buffering power (β) and ionic strength (I) of Rilbe's system in Fig. 5. His data were re-calculated with our computer program by assuming acetate to be an Immobiline with pK 4.61 as follows: buffering ion = 6.52 mM in both chambers; titrant (Immobiline of pK 12), 1.06 mM in the acidic chamber and 5.19 mM in the basic chamber. The acetate concentration was selected so as to give an average β value of 3 mequiv. l^{-1} pH^{-1}. Again, our computer simulation predicts that Rilbe's gradient is fully compatible with a properly behaving IEF system.

3. DISCUSSION

Modern IEF became a reality when buffers became available that met Rilbe's fundamental requirements: (a) of being amphoteric, so that they could reach a steady state by being titrated to a pH level ensuring "zero net-charge"; and (b) of being "carriers", *i.e.*, of being good conducting and good buffering species at their p*I* values. After about 25 years of conventional IEF, the four modes of non-amphoteric buffer IEF that we have summarized and compared here clearly demonstrate that the system no longer depends on amphoteric species. However, a properly performing IEF system still has as an absolute requirement the concept of a "carrier", which requires that the chemicals used to generate and stabilize the pH gradient behave as "good buffers" and "good conductors". The systems discussed in sections 2.2–2.4 fulfill this fundamental requirement; in these three instances, in fact, quasi-linear pH gradients are generated by titrating weak acids or weak bases symmetrically around their respective p*K* values, where they automatically provide the much needed buffering capacity and conductivity. In the Chrambach "arrested stack", the system breaks down because the potential buffers are allowed to be stripped electrophoretically of counter ions and thus to collect in strongly acidic anodic layers or strongly basic cathodic zones, where they are deprived of their buffering and conducting powers. While it is true that in Chrambach's system a natural pH gradient can still form (it is in fact a pH gradient generated by a stack of moving boundaries, like in isotachophoresis), it can hardly be controlled (for lack of buffering power) and it can never be assumed to become stationary (for lack of immobilization). In fact, the so called "arrested stack" created by protonation of acids and deprotonation of bases, is in fact never completely arrested; it could only be so when the current in the system becomes zero, but at this point it would be meaningless still to speak in terms of "electrophoresis" (which, by definition, requires a current to be flowing through the system). As for the other systems, although all three are based on sound and correct hypotheses, they are markedly different in operational terms. Bier *et al.*'s system is subject to two inherent disturbances: (a) migrational instability (the parameter ρ will very rarely be unity); and (b) diffusional instability (decay of the boundaries). The two instabilities are additive and will ensure ultimate decay of the pH gradient. As for Rilbe's system, here again what theory predicts and what practice can achieve rapidly come into conflict: the predicted pH courses are only established in the absence of an internal liquid flow (*i.e.*, inside the electrophoresis cell); unfortunately, there is always a net liquid flow within electrolysers, and this induces exponential, and slowly decaying, pH gradients.

About 10 years ago, Catsimpoolas[21] predicted that conventional IEF had the potential for measuring the diffusion coefficients (D) and mobility slopes (du/dpH) of focused proteins. However, the strong discrepancies between theoretical and experimentally measured values induced him to call for the synthesis of "second generation ampholytes", which could not only provide highly stable pH gradients, but also uniform conductance and concentration distribution courses throughout the separation path. Perhaps present-day IPGs represent just the ideal system envisaged by Catsimpoolas: they have certainly overcome all the drawbacks of conventional IEF and they truly provide for a medium of unlimited stability. However, we prefer to end with a note of caution: even such a highly refined and extremely successful

method as IPG, while superbly fit for performing IEF, will still not allow proper measurements of D and $du/d(pH)$, because the protein will form a salt with the matrix as it begins to move away from the pI zone (possible even in the pI region).

4. SUMMARY

Four potential modes of isoelectric focusing in non-amphoteric buffers are evaluated: (a) "stack" or "train" of free bases or acids "arrested by a deprotonation or protonation mechanism", respectively (Chrambach); (b) "chemically bonded" (immobilized) pH gradients (Righetti *et al.*); (c) "physically bonded" or "quasi-immobilized" pH gradients (Bier *et al.*); (d) steady-state rheoelectrolysis (Rilbe). The first is based on a "catastrophe" theory, *i.e.*, it confines the buffers in a pH region where they can create a pH gradient by an isotachophoretic mechanism, but where they do not have sufficient buffering capacity to stabilize it; no true isoelectric focusing can ever be achieved with this system. The last three are based on sound and well defined theories; however, at present, only system (b) (immobilized pH gradients) has proved to be a simple and reliable technique, easily transplantable in any laboratory. Bier *et al.*'s and Rilbe's approaches require complex and elaborate experimental set-ups and strict adherence of laboratory practice to a set of physical laws governing the system. In practice, owing to the divergence of experimental approaches from idealized physical equilibria, the last two approaches appear still to be far away from daily laboratory work.

5. ACKNOWLEDGEMENTS

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CHREV. 192

AUTOMATION OF ANALYTICAL ISOTACHOPHORESIS*

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CONTENTS

1. INTRODUCTION

In isotachophoresis (ITP), sample components separate by forming discrete, consecutive zones with homogeneous concentrations. This system reaches a steady state, in which each sample zone moves with constant velocity, the zone length being proportional to the amount of a sample constituent. The boundaries between adjacent zones are characterized by sudden changes in a number of physical properties which allows quantitation by zone length measurements[1–5]. This behavior of electrical transport systems is unique and not duplicated by any other separation technique. Although many publications have praised the high resolution of ITP, other methods, such as high-performance liquid chromatography (HPLC), have been more rapidly explored and introduced in analytical laboratories. Commercially available ITP instruments are very expensive and not easy to operate by a lab technician. In addition to these obstacles, the whole methodology, mainly the choice of a suitable operational system for a specific separation problem, has not yet been compiled in a straightforward way. The superiority of ITP compared to any heterogeneous type of chromatography or any type of field-flow fractionation technique, however, has been fully demonstrated in recent years. Important contributions include: (i) the theoretical description of the isotachophoretic separation process with a model based on migration only[6–8]; (ii) the experimental verification of the separation scheme[9,10]; (iii) the understanding of the zone order in ITP[11]; (iv) computer simulations whose results

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are consistent with the findings in (i)[12] and (iii)[13] and provide further insight into the features of the boundary structure[14]; and (v) the development of high-resolution detectors which monitor either universal or specific properties of the separatand pattern.

The automation of the ITP process is necessary for the adoption of this technique as a routine analytical laboratory methodology. It has been clearly established in the past decade that digital electronics has an enormous amount to offer to analytical chemists. The advent of versatile and inexpensive microprocessor systems in conjunction with the demonstration of the advantages of ITP can be expected to cause a revolution in the analytical use of ITP. The evolution of an ITP separation as well as the migrating steady-state zone pattern provide the prerequisites for automation. Transient- and steady-state zone distributions can be monitored by a plurality of equidistant detectors along an ITP column (array detector[10,15–17]), or by scanning the separation trough repeatedly with a moving detector[18]. This gives a computer interpretable criterion for the steady state, which is a mandatory condition for automation. Much information of analytical relevance is lost with single detectors placed at the end of the separation space, the method used in all commercial instruments.

Prior to considering possible future developments in the field of automated analytical ITP it is pertinent to reflect on the author's strong links which have generated many of the ideas and predictions formulated in this article. In particular he would like to acknowledge the very significant contributions of E. Schumacher and D. Arn, University of Bern, Switzerland as well as of M. Bier, R. A. Mosher and N. B. Egen, University of Arizona, Tucson, AZ, U.S.A.

This lecture reviews the basic features of automation of analytical ITP. Experimental setups consisting of narrow bore tubes (capillaries) are considered which are self-stabilized against thermal convection. Sample detection in free solution is discussed by listing the detector systems presently used or expected to be of potential use in the near future. The combination of a universal detector measuring the evolution of ITP zone structures with detector systems specific to desired components is proposed as a concept of an automated chemical analyzer based on ITP. In addition possible miniaturization of such an instrument by means of microlithographic techniques is discussed.

2. DETECTION OF ITP ZONES

In principle many of the detection methods developed for HPLC can also be employed for ITP. In addition, unique principles can be used which rely on the constant electric current flow through the separation capillary. ITP zones are therefore characterized by a distinct, constant voltage gradient, resulting in a stepwise electric field change across their boundaries. Conductivity and temperature profiles are of comparable shape. The transition between a pair of sample components can be further marked by a sudden change in a number of other physical properties, such as refractive index, pH and absorbance. A universal detector allows the monitoring of the whole zone structure, the magnitude of the sensor response being a function of the net mobility of the sample constituent[2,3]. The information derived, therefore, has a stepwise character. Methods include the measuring of the potential gra-

TABLE 1

DETECTION SYSTEMS FOR ISOTACHOPHORESIS

Detection system	*Performance*	*Scan*	*Array*
(a) Universal methods			
Potential gradient	Microsensing electrodes in contact with electrolyte		×
Conductivity	Microsensing electrodes in contact with electrolyte		×
Conductivity	Microsensing electrodes without electrolyte contact	×	×?
Temperature	Thermocouple, thermistor		×
Thermal radiation	Heat radiation sensor	×	
UV–VIS absorbance*	Optical micro cell	×	×
Fluorescence quenching**	Optical micro cell	×	×
(b) Optical methods			
Polarimetry	Optical micro cell		×
Schlieren-interferometry	Optical micro cell		×
UV–VIS absorbance	Optical micro cell	×	×
Fluorimetry	Optical micro cell	×	×
(c) Radiometric methods	G.M. counter	×	
	Scintillation detection	×	×
(d) Electrochemical methods			
Ion-selective electrodes (pH?)	Microsensing electrodes in contact with electrolyte		×
Voltammetry	Microsensing electrodes in contact with electrolyte		×

* If applied with absorbing counter component.
** If applied with fluorescing counter component.

dient[2,3,10,15–17,19–23] and the conductivity[2,24] with sensing microelectrodes which are in direct contact with the electrolyte, the determination of the conductivity with sensors outside of the separation column[25] and the detection of the temperature profile with thermocouples or thermistors[2,26] and with heat radiation (IR) sensors[27]. Optical detectors are also considered as being universal if the common counter constituent, but not the sample, can be monitored[2,17,28]. The signal of pH sensors is proportional to the net mobility of the sample components if buffer-free operational systems are employed[1,17,23].

Other methods allowing the visualization of the majority of boundaries include polarimetry, Schlieren-interferometry or ultrasonic imaging. The magnitude of the signals derived therefore are not related to the net mobility of the sample. A non-continuous response is recorded. This is also typical with the use of specific detectors which permit the localization of a desired sample constituent in a complex isotachopherogram. Specific methods include UV–VIS spectrophotometry[2,29], fluorimetry[28], radiometry[30], potentiometry with ion-specific electrodes[2] and voltammetry[31]. Table 1 summarizes the methods of detection currently employed in analytical ITP, together with those of potential use in the near future. Their feasibility for the con-

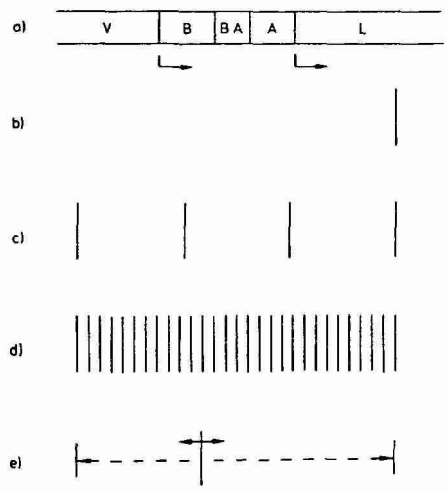

Fig. 1. Schematic presentation of detector arrangements along an ITP column. (a) Separation trough with transient zone structure. L refers to the leading zone, V to the displacing electrolyte and BA represents a transient mixed zone composed of components A and B. (b) Single detector placed at the end of the trough. (c) Four detectors along the column. (d) Array detector as a plurality of equidistant detectors. (e) Single detector adapted for movement (scan detector).

struction of scan and linear array detectors is also included in this list, since location and arrangement of sensors is of crucial importance for a suitable detection and therefore for automation of this analytical technique. A schematic representation of various detector arrangements along an ITP column is given in Fig. 1.

3. MONITORING OF THE SEPARATION PROCESS

The continuous monitoring of the evolving zone structure constitutes the most straightforward approach for the visualization of the separation process. With a plurality of equidistant detectors along the column (array detector as depicted in Fig. 1d) transient- and steady-state zone distributions can be observed. When measuring a general physical property the time development of each zone boundary can be monitored, including the vanishing of mixed zones. An apparatus incorporating a potential gradient array detector as one wall of the isotachophoretic separation trough was constructed at the University of Bern, Switzerland[10,16,17,32]. The sensor array consists of an ordered array of 256 detection electrodes over a length of 10 cm, permitting almost simultaneous measurements of the electric field along the column. Fig. 2 shows the anionic boundary between 2.5 m*M* hydrochloric acid, the leader, and lactic acid as terminator while moving across part of the detection window. The anode is to the right. Data points of 8 scans over 177 detection channels are displayed. They are corrected for irregularities of the crossectional area of the separation trough[33]. The consecutive scans are shifted by an offset in the *y*-axis for their presentation in one graph. This experiment clearly documents the steady-state behavior of this boundary. Fig. 3 shows corrected data points of scan 11 with maleic acid, tartaric acid and malic acid, 3.5 nmol each, as sample components. The operational system is identical to that of Fig. 2. The presented data are machine treated as dis-

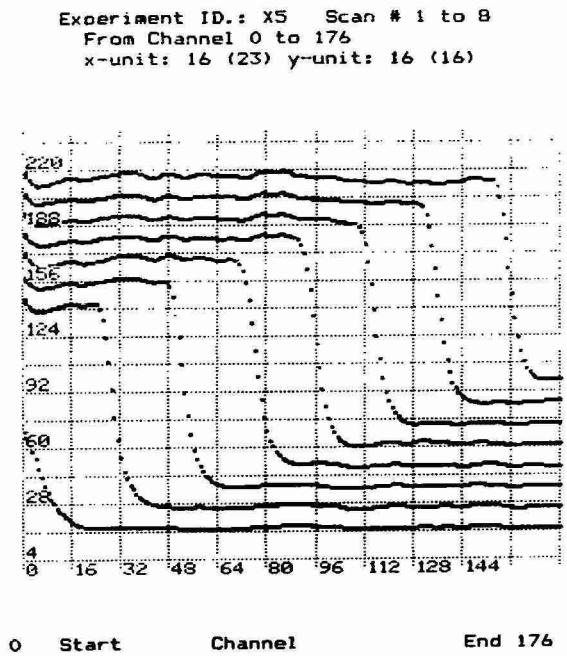

Fig. 2. Evolution of a steady-state anionic boundary as monitored with the potential gradient array detector. 2.5 m*M* hydrochloric acid and 10 m*M* sodium lactate were used as anolyte and catholyte respectively. Consecutive scans were taken every 46.5 sec under a constant current of 100 μA. Machine treated data points of scans 1 to 8 along a part of the detection window are displayed.

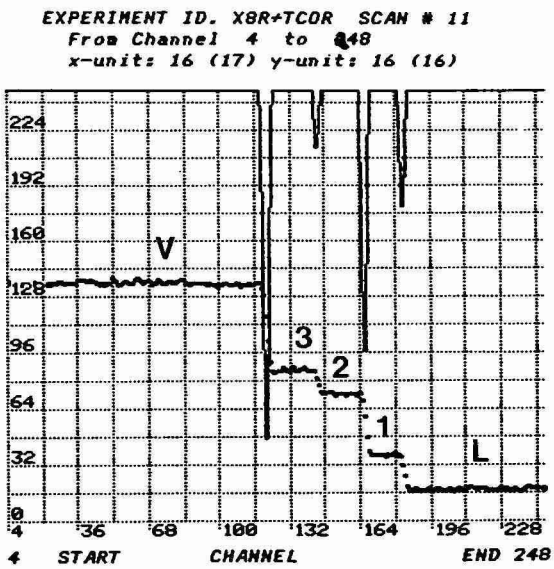

Fig. 3. Data of scan 11 representing the electric field profile after 566 sec of current flow across an isotachopherogram with maleic acid (1), tartaric acid (2) and malic acid (3). The leading zone is denoted by L and the displacing electrolyte by V. Data points and first derivative from top are machine treated. The output protocol provides boundary locations at detector channels 182.46, 165.37, 143.61 and 119.89. Zone heights are 17.44 (L), 36.99 (1), 71.91 (2), 85.76 (3) and 135.34 (V)[33].

cussed by Schumacher *et al.*[33]. Zone lengths are given by the number of detection channels occupied by the zone during the scan. Note that a five-fold decrease of the injected amount of sample still would be detectable with the sensor array. Zone heights are in digital units allocated through the 8 bit analog to digital converter. That the steady-state zone structure was attained within the detection window under the given conditions is shown in ref. 32. An on-line evaluation of data, *i.e.* the comparison of consecutive scans during the experiment is currently under investigation[34,35]. This provides the last step necessary for the completion of the new concept for automated monitoring of the isotachophoretic separation[15].

An alternative procedure represents the use of a single detector adapted for movement along the separation trough (Fig. 1e). By repeatedly scanning and continuously measuring a universal physical property almost instantaneous profiles could be obtained. A necessary requirement is that the scanning speed of the detector has to be much higher than the electrophoretic velocity of the zone pattern. No such detector has been constructed yet. Possible candidates include the high-frequency contactless conductivity detection and the heat radiation detection. Data processing in this operational mode is identical to that for the array detector referred to above. It is important to realize that no correction of steady-state zone lengths to equal measuring time is necessary if current density and scanning rate are constant. Every zone is elongated in the laboratory frame proportional to its true length in a moving frame of reference. This is, of course, only correct in a uniform velocity field, which is realized in steady-state ITP. The proportionality is then constant and will drop by calibration from further consideration.

Information about the steady state can also be obtained by comparing the zone patterns from at least three completely different locations of the separation trough (see Fig. 1c). Such a multi-sensor procedure for measuring the ITP spectrum at different instants can easily be automated with computer control. Simple algorithms allow the evaluation of the digitalized detector responses. This mode of operation has been mentioned in the literature and can be performed using the apparatus with multichannel zone detection[10,16].

Optical multichannel array detectors for the continuous monitoring of specific ITP zones have been proposed[17] but not yet constructed. The application of a linear fiber-optic developed for facsimile transmission[36] could be an interesting approach in this area. Linear diode arrays (LDA) or charge coupled devices (CCD) constitute solid state imaging devices without any mechanical parts and are, together with ultrasonic imaging systems, potentially useful in ITP. The utility of an optical scan detector in electrophoresis has been demonstrated by Hjertén[18]. His quartz tube apparatus, stabilized by rotation about its longitudinal axis, is scanned repeatedly at two UV frequencies. The ratio between the two wavelengths is recorded. By such automatically repeated scans one can follow separation of UV absorbing sample components throughout a run and determine if or when a steady state has been attained.

With just one detector placed at the end of the separation column (see Fig. 1b) it is not certain if the steady state, *i.e.* the number of boundaries (or zones) and/or the constant length of each zone, has been attained at the time of detection. Each experiment must be repeated with different amounts of the sample components followed by a careful comparison of the detected zone structures. This mode of opera-

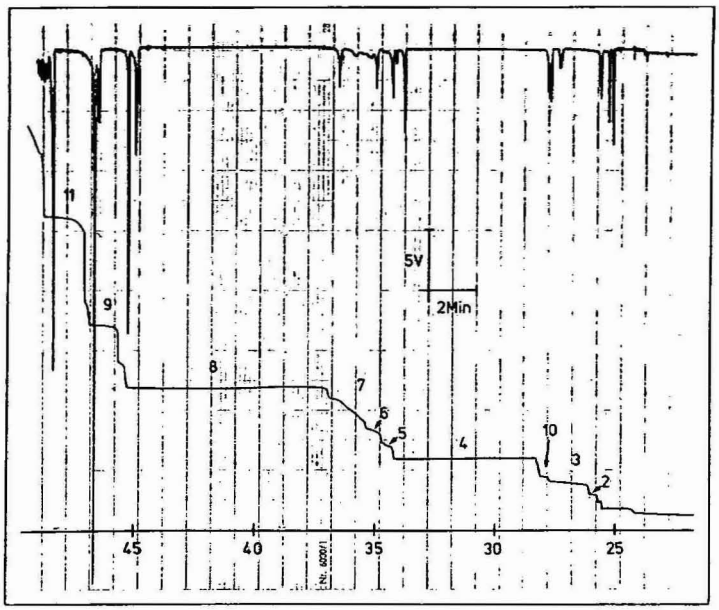

Fig. 4. Analysis of 1.5 μl of a Swiss white wine. A current of 100 μA was applied. Denoted zones: (2) pyruvic acid, (3) phosphoric acid, (4) tartaric acid, (5) citric acid, (6) malic acid, (7) galacturonic acid, (8) lactic and gluconic acid, (9) succinic acid, (10) α-ketoglutaric acid, (11) acetic acid. The x-axis scale is the time in min after current application.

tion is implemented in all commercial ITP instruments and represents the simplest procedure for the visualization of an electrophoretic zone structure. Its disadvantages are obvious and have been reported extensively. Automation of this mode would not only be inelegant but would not exploit the tremendous capabilities of digital electronics associated with microprocessors.

4. AUTOMATED CHEMICAL ANALYZER BASED ON ISOTACHOPHORESIS

It has been shown that the isotachophoretic separation can be followed with an array detector measuring a general physical property. The ability of an ITP instrument to separate and detect compounds in complex mixtures generally far exceeds that instrument's ability to provide enough information for the identification of those components. Although the magnitude of the universal sensor signal is qualitative information it is often not easy to assign each detected zone to a specific substance. Correlation with standard solutions can be hazardous due to the formation of steady-state mixed zones, produced by constituents which have equal or similar net mobilities in a given operational system. The pherogram presented in Fig. 4 demonstrates the inability of the potential gradient sensor to reliably identify all detected zones. A volume of 1.5 μl of a Swiss white wine was analyzed with hydrochloric acid as leader (pH = 2.60) and hexanoic acid as terminator. To enhance the separation capacity of the given column a concentration cascade[37] of the leading electrolyte was utilized (32.5 μl of 10 mM hydrochloric acid[10]). Detection occurred at channel 250. The denoted zones were determined by the addition of internal standards and by

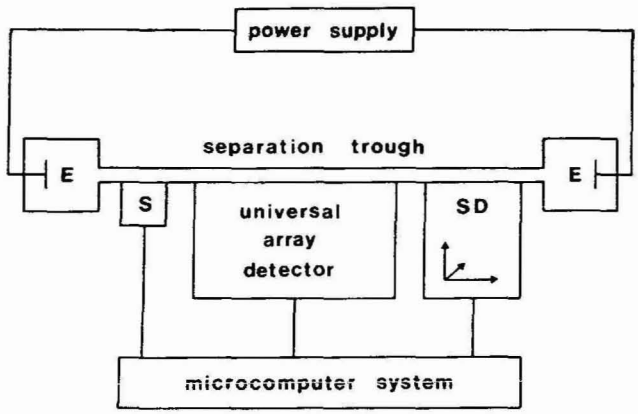

Fig. 5. General block diagram of a chemical multicomponent analyzer based on automated analytical isotachophoresis. E = electrode compartments; S = sample inlet system; SD = specific detector module.

comparison of the zone heights with test samples. Note that in the given operational system lactic acid and gluconic acid were found to have equal net mobilities. It is certainly difficult if not impossible to identify all zones detected in this acid-spectrum. Additional sensors which show specific response of desired components have to be used. They improve considerably the overall performance of ITP: faulty assignments are minimized, unknown components can be identified and further off-line fractionation techniques[38] do not have to be used. The great variety of suitable specific detector systems (see Table 1), together with microprocessor control and chemometrical software, provide flexibility for a wide range of applications. Spectroscopic techniques appear to be the most popular. The construction of simple, interchangeable modules (or module arrays) is required, to allow an easy and quick selection of an appropriate method.

The overall concept of a chemical analyzer based on ITP comprises therefore three important parts, as shown schematically in Fig. 5: (i) a universal detector for the monitoring of the separation process; (ii) a second (or more) detector(s) for the specific detection of selected sample components; and (iii) a microprocessor system for control of the entire apparatus with multiple detection systems, for data storage and data handling as well as for database dictionaries with instructions for the user. Advantages and the necessity of part (i) have been discussed earlier in this manuscript and elsewhere[8,10,16]. It is worthwhile to add some comments pertaining to specific detection systems as well as to the computer system.

UV photometric detection has been employed for a number of years and is included in commercial capillary type apparatus. The combination of this selective detection technique with high-resolution conductance or electric field measurements has been demonstrated as being extremely helpful for the evaluation of complex isotachopherograms[2]. Two sensors placed in series at the end of the separation column were used. New optical microcells for dual-wavelength absorption detection[29] or simultaneous fluorescence (or fluorescence quenching) and absorption detection[28] have been designed and successfully applied to ITP analyses together with computerized signal storage and processing. Data reduction on the basis of signal ratios, fea-

turing greater sensitivity and selectivity has also been developed. Fixed-wavelength detectors have been primarily used. This is a major limitation since only one or two wavelength resolution elements are registered at a time. In HPLC however rapid-scanning UV–VIS photometric detection based on electronic, electromechanical or multichannel devices is already in use[39,40]. The gathering of complete absorbance or fluorescence spectra in the order of msec, *i.e.* the simultaneous detection of a large number of spectral resolution elements, adds an additional dimension of information. The data in digital form can be readily stored, manipulated and presented in any desired format, making it possible to obtain three-dimensional chromatograms within the time needed for a single run. Optical multichannel detectors based on LDA, silicon intensified target (SIT) vidicon or CCD are most promising for the rapid acquisition of spectral data.

Radiometric detectors can be used for the analysis of radioactive and radio-labelled ionic compounds. A first approach achieved by Kaniansky *et al.*[30] highlights the advantages of this specific detection principle. There is no doubt that this technique will be very valuable in clinical, biomedical and biochemical applications. Little attention has been paid to electrochemical detection so far. The use of electrodes with specific coatings has been discussed by Everaerts *et al.*[2]. Ion-specific sensors based on ChemFET technology[41] (pH?) are yet to be applied for detection in electrophoresis. Voltammetry remains another detection technique to be introduced. Electrochemical detection is widely used for HPLC because of its excellent sensitivity for many electroactive compounds. The most commonly employed constant potential mode has only modest selectivity. Transient techniques, such as pulse, alternating-current or square-wave voltammetry, however, have been reported to be very useful in liquid chromatography if used with microprocessor-based instrumentation[42]. The simultaneous recording of five or more chromatograms on different time scales with different sensitivities and the recording of complete current–voltage curves at all points in time have demonstrated the advantages and great sensitivity as well as flexibility of these techniques. Rapid potential scanning procedures allow the gathering of entire voltammograms in the order of seconds[42–44]. Three dimensional data presentation is therefore possible which is comparable to that obtained with optical multichannel detectors.

The microcomputer represents the cybernetic center of the apparatus. Since the detailed approach to the steady isotachophoretic state is followed by the computer, an automated decision about the completion of the separation is provided. Separation time and separation location are thereby known. As soon as the criterion for stability is fulfilled, the microcomputer takes several scans over the total component spectrum to ensure a specified precision of zone length. The calculation of zone lengths from the length and decomposition rate of mixed zones represents an alternate way to obtain insight into the steady state zone structure. This transient approach applies nicely for simple systems but fails for complex configurations where mixed zones contain several components. Data from the array detector are processed as described by Schumacher *et al.*[33] and Arn and co-workers[34,35]. Data from specific detectors are digitalized and compared with those of reference components which are stored from calibration runs in the computer library. The computer assigns each detected zone to a specific substance based on the information gathered from both detector systems. It is important to realize that proper analytical self testing and elaborate data analysis is performed unnoticed by the user.

The aforementioned tasks and advantages of a computer system in an automated instrument are not complete without the establishment of a computer database containing methodological instructions for the user. The wide variety of operational systems suitable for ITP analyses should be summarized and stored in the computer. Such a compilation enables the user to find appropriate leading and terminating electrolytes as well as the specific detection system suitable for a specified analysis. This information enables an untrained user to perform successful runs. Similar libraries have been found useful in various areas of analytical chemistry, such as in automated titrimetry.

5. DISCUSSION

Many analytical procedures based on wet chemistry are extremely time-consuming and consequently can only be economically justified if automated. Although various steps in a manual wet chemical method can be mechanized, the objective should be to automate the entire procedure. Automated ITP provides many advantages and is an alternative to automatic titration[15]. An array detector measuring a general physical property provides insight into the evolution of the whole zone spectrum. An ideal apparatus requires more than just the capability of following the separation process together with a computer for data storage and handling. It would also require a second detecting system specific to desired sample components. The combination of two (or more) detector systems would be most suitable in routine analysis if automated with computer control. Such a device would provide the basis for an automated chemical multi-component analyzer. The decision making process of a computer system is based upon a comparative procedure. In automated ITP the computer software would compare: (i) the dynamics data of subsequent scans for the validity of the isotachophoretic state at each boundary and (ii) the universal and specific sample responses with a previously compiled data base. Quantitation through zone length measurements is achieved by comparing the detected distance between two boundaries with those from preregistered calibration runs or by the method of standard addition. The availability of methodological instructions for the user, organized as a computer library, would represent another feature of the analyzer.

The potentialities of new detector systems have been discussed on a broad basis. In particular fast scanning optical and swept-potential electrochemical detectors allowing three dimensional projections of data are proposed as important methods for the more complete identification of ITP zone structures. It will certainly take some time for their introduction in ITP. In the meantime it is hoped that this manuscript stimulates new activities in this field of chemical analysis.

The isotachophoretic sorting process is a focusing method in the sense that it is capable of concentrating a dilute component into a narrow zone. Conversely, a concentrated component in the sample will form a relatively wide zone. In both cases there is no further change of the zone length when a steady state is reached, *i.e.* when the spectrum of contiguous sharp zones whose boundaries migrate with equal velocity is fully established. The width of ITP boundaries is dependent upon experimental parameters, such as the current density and the chemical–electrochemical properties of the components involved. It can be predicted by simple models[14] and is usually in the order of micrometers. Each of these unique features is important for auto-

mation of this electrophoretic mode. They also provide favorable scaling laws, *i.e.* an estimate for suitable miniaturization of the detectors and the separation columns, and would allow instrumentation to be shrunk, possibly to the dimensions of a printed circuit board. Technologies developed for microelectronics, namely microlithography and chemical etching, as well as the advent of versatile and inexpensive microprocessor systems provide the means for the construction of new instruments. The introduction of miniatured gas chromatographs[45] and of microconduit flow injection analysis[46] are examples of the straightforward applicability of such methods. Based on these examples, and the experience obtained with the potential gradient array detector, the design steps of a new, more versatile ITP analyzer are given. Its construction should be feasible in the foreseeable future.

The analysis of wine presented in this paper (Fig. 4) illustrates a field of application for ITP as a multicomponent analytical methodology. The determination of acetic (zone 11), succinic (zone 9) and α-ketoglutaric acid (zone 10) allows the prediction of the quality of the wine because of the disagreeable taste of those components. The ratio of the concentrations of certain acids in a wine further provides a method to conclude its fermentation process. ITP is not only an excellent method to monitor simultaneously the entire acid-spectrum of a wine; similar determinations can be done with other beverages (beer, soda, milk, fruit juice), food extracts, body fluids (serum, saliva, urine, sweat), fermentation solutions in genetic engineering, tissue culture media for hybridoma technology and nutrient solution for the hydroponic industry as well. An automated ITP instrument would certainly be very useful in these and other fields of application.

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7. SUMMARY

A review of progress in the field of computer-aided methods for the monitoring of the isotachophoretic separation process and the steady-state zone structure is presented. Sample detection in free solution and the potential utility of three dimensional projections of data from universal and specific detectors are further discussed in the context of a proposed concept of a multicomponent chemical analyzer based on automated analytical isotachophoresis. The analyzer yet to be designed would benefit from favorable scaling laws, which would permit the utilization of microlithographic techniques for its construction. This would lead to miniaturized instrumentation in capillary electrophoresis.

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