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EVALUATION OF THE OPTIMIZATION POTENTIAL IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS OF OPTICAL ISOMERS WITH SWOLLEN MICROCRYSTALLINE CELLULOSE TRIACETATE

ANDREAS M. RIZZI
Institute of Analytical Chemistry, University of Vienna, Währingerstrasse 38, A-1090 Vienna (Austria)
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SUMMARY

Quantitative data concerning the effect of temperature, solvent composition and flow-rate on the enantioselectivity, efficiency and retention for optical isomers on swollen crystalline cellulose triacetate are reported. Based on these data, the optimization potential of the named variables for the separations of optical isomers is discussed with respect to the chromatographic resolution as well as to the analysis times and the detection limits for the analytes. The trends observed can serve as guidelines for the derivation of optimization strategies.

INTRODUCTION

Triacetylated cellulose material has been used for several years as a stationary phase for the chromatographic separation of optical isomers[1–18]. Due to the current high interest in the role of optical isomers in biochemical processes, separation techniques for enantiomeric compounds are developing rapidly. This has led also to a renaissance in the use of cellulose triacetate (CTA). Its chemical structure allows various types of interactions with functional groups commonly encountered in drugs, thus enabling chiral recognition and discrimination for a variety of analytes of different structural types and sizes. This explains the wide field of applications of CTA chromatography to pharmaceutically active substances, which have been reviewed recently by Blaschke[12] and Shibata *et al.*[14].

CTA can be used either in a swollen microcrystalline state or as a layer coated on porous silica particles. Both types have quite different chromatographic behaviours, especially stereoselectivity and efficiency. The differences in stereoselectivity have been discussed by Shibata *et al.*[14].

This paper deals with the evaluation of the optimization potential of different variables in chromatography with swollen microcrystalline cellulose triacetate (swcr-CTA). The dependence of the plate height, enantioselectivity, capacity factors and the resolution on the eluent composition and on the process variables, temperature and flow velocity, has been investigated systematically. The implications of these findings are discussed for a rational optimization of the chromatographic separations.

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A thorough optimization of a separation may be of enhanced interest for routine analysis methods or where preparative scale separations are performed.

EXPERIMENTAL

Apparatus

Chromatographic experiments were carried out using a high-pressure liquid chromatographic pump (Model L-6200 intelligent pump; Merck-Hitachi, Tokyo, Japan), a syringe-valve injector (Model 7161; Rheodyne, Cotati, CA, U.S.A.) equipped with a 20-μl loop, a column oven (Model 655A-52, Merck-Hitachi) and an UV detector (Model L-4000, Merck-Hitachi) connected to an integrator (Model D-2000 chromato-integrator, Merck-Hitachi).

Column

A prepacked column (250 mm × 10 mm I.D.) filled with swcrCTA with a mean particle diameter of 10 μm was used (Hibar®; E. Merck, Darmstadt, F.R.G.).

Reagents and samples

Organic solvent components were obtained from E. Merck. Methanol and hexane were of LiChrosolv® quality, absolute ethanol of p.a. quality. Water used for the eluent preparation was distilled twice from a quartz apparatus and additionally purified by passing through a RP-8 column. The eluent mixtures were premixed and degassed in an ultrasonic bath.

The analyte samples were obtained in the highest purity grade available or were received in an highly purified state as gifts from synthesis laboratories.

Procedure

All the data refer to isocratic elution at the given temperature. After establishment of the thermal equilibrium, the constancy of retention data was about $\pm 1\%$. The void volume of the column was estimated from the retention volume of the system peaks of injected water, methanol or propanol and was approximately 15 ml. All the calculations of the capacity factors are based on a void volume of 15.00 ml for all solvent mixtures. UV detection was performed at 254 nm.

BASIC MODEL

Retention mechanism, capacity factor and stereoselectivity

swcrCTA differs significantly in its adsorbent characteristics from other packing materials. As this material provides different types of adsorption sites, different mechanisms of adsorption seem to exist. The retention of an analyte is determined by its structure in a way which is not completely understood and for which only some empiric rules can be given.

Interactions both between polar structures and between non-polar structures seem to be important. Obviously, the molecular volume plays an important role in this respect. For molecules with sterically large structures, like *tert.*-butyl groups, an exclusion from most of the adsorption sites is observed, *e.g.*, for tri-*tert.*-butylbenzene. Similarly, in ethanolic eluents, charged organic analytes are eluted before the column

void volume[19]. For non-polar aromatic and polyaromatic hydrocarbons the retention decreases with increasing size of the molecule. Generally, however, the retention is not simply correlated with the molecular size. Obviously, the configuration of the analytes plays an important role in the retention, otherwise this material would not be of great use for enantiomeric separations.

Prior to the presentation of the results a model concerning the adsorption mechanism is briefly discussed which can serve as a basis for the interpretation and discussion of the experimental data in the next section. The model has been derived to a significant extent from peak dispersion data for swcrCTA adsorbents[19,20]. Those investigations reveal that with respect to the mass-exchange kinetics, at least two (but maybe more) different types of adsorption sites are operative: "quick"-type and "slow"-type sites. These types differ in the kinetics of the adsorption/desorption process, mainly due to the different diffusion velocities very near to the adsorption sites. The adsorption sites differ also in their availability for different analytes and probably in the strength of interaction with polar groups in the analyte molecule. (The existence of a mixed adsorption mechanism, *i.e.*, of different types of sites with respect to the type and strength of interaction, has been proposed by Scharf *et al.*[13]. It is likely that the differences in the kinetic features and in the types of interactions are correlated: the sites which form stronger interactions with polar groups in the analytes seem to be of the "slow"-type[19].

Concerning the peak broadening process, the observed plate height is predominantly dependent on the kinetics of the adsorption/desorption proces. Hindered diffusion to (and at) narrow (maybe "channel"-like or "cavity"-like) structures of the swollen absorbent is probably responsible for the slow kinetics, which is observed for most analytes. The plate height of an analyte is determined (i) by the accessibility of the adsorption sites which influences the velocity of motion of the analytes to and at the sites, and (ii) by the relative contributions of "slow" (narrow)- and "quick"-type (broad) sites to the retention of the analyte. The most important parameter influencing the plate height of the analytes is therefore their molecular structure, since the structure determines the steric size and the types of interactions predominantly formed, and thus the diffusion velocity and the type of sites at which the analyte is preferentially adsorbed. The plate height is therefore not simply correlated with the capacity factors of the analytes. Any change in the accessibility and in the availability of the different types of sites, *e.g.*, by changes in the eluent composition, influences the overall plate height.

The retention of analytes is determined by the activity coefficients of the analytes in the mobile phase, and by the availability of the different adsorption sites. The availability depends on the swelling state of CTA and on the strength of the competitive adsorption effects of the solvent components at the adsorption sites. With the exception of the changes in the swelling state, this behaviour is analogous to other types of adsorption chromatography. Since both types of interactions, namely those between polar structures and those between non-polar structures, are operative, polar and non-polar solvent components act as competitors for adsorption. As regards the strength of the competitive effect, the size of the solvent molecules seems to play a major role. The solvent composition also influences the swelling state of the swcrCTA packing, and thus the number of different adsorption sites available. Owing to all these facts, no simple dependence of the capacity factors on the "hydro-

phobicity" and the "polarity" parameters of the eluent mixture is found, in contrast to reversed-phase chromatography with alkylsilica adsorbents and aqueous mobile phases.

For the steric discrimination of enantiomers, narrow adsorption sites seem to be decisive. Most probably these sites are identical with those observed as "slow"-type sites from the point of view of the kinetics of mass exchange. The solvent composition affects the availability of these narrow sites either by the competitive adsorption of solvent components or by influencing the swelling state of the packing material. In these ways the solvent composition has a strong influence on the stereoselectivity.

The dependence of the capacity factor, κ, enantioselectivity coefficient, α, and theoretical plate height, H, on temperature, eluent composition and flow-rate is reported in detail in the following section.

RESULTS AND DISCUSSION

For the optimization of separations of enantiomers in high-performance liquid chromatography (HPLC) the most frequently used criterion is sufficient resolution of the optical isomers, obtained in the shortest possible analysis time, with the lowest possible sample dilution. In this paper the following definition of the chromatographic resolution, R_S, is used

$$R_S = (\alpha - 1) \cdot \frac{\kappa_I}{1 + \kappa_I} \sqrt{\frac{N_I + N_{II}}{2}} \tag{1}$$

where κ denotes the capacity factors of the analytes, the indices I and II refer to the first and second eluted isomer, respectively, α denotes the enantioselectivity coefficient and N the plate number with respect to the indicated enantiomer.

The reason for using the mean value of N in eqn. 1 is the observation that the plate numbers of the two isomers are most often considerably different when using sweetCTA packings. This allows (i) the correlation to be kept between the numerical values of R_S and the separation effect obtained more or less unchanged in the usual manner (for symmetric peaks of equal height, a R_S value of 6 means approximate "baseline separation"), and (ii) calculation of the minimum plate number, $\bar{N}_{min}$, which is necessary to obtain a certain resolution, in a simple way. This would not be as simple when applying a more fundamental equation[a] for the resolution of peaks with strongly

[a] The chromatographic resolution of two peaks with very different plate numbers is defined in a more appropriate way by

$$R_S = \frac{t_{Rj} - t_{Ri}}{\bar{\sigma}_t}$$

where $\bar{\sigma}$ is the mean standard deviation of the two peaks. Substituting the retention time and the peak standard deviation by operational parameters leads to:

$$R_S = (\alpha_{ji} - 1) \cdot \frac{2\kappa_i}{(1+\kappa_i)\sqrt{N_j} + (1+\kappa_j)\sqrt{N_i}} \sqrt{N_i N_j}$$

different peak widths. However, for the discussion of the trends in resolution and analysis time, in this paper, the approximation of eqn. 1 is acceptable.

The minimum analysis time is defined in this context by

$$t_{R,min} = (L_{min}/u)\,(1+\kappa_{II}) = (\bar{H}\bar{N}_{min}/u)\,(1+\kappa_{II}) \quad (2)$$

where H denotes the theoretical plate height, L the column length and u the linear flow velocity.

Optimization of the separation can be carried out by tuning at least one of the three decisive chromatographic parameters, α, κ, and/or N. Thus, the knowledge of the quantitative dependence of these chromatographic parameters from the variables, eluent composition, temperature and flow-rate, is a prerequisite for any rational optimization.

The chiral analytes shown in Table I were used for this investigation.

Optimization potential of the temperature

The influence of the temperature on the capacity factor, the enantioselectivity and the plate height is given in Table II. The capacity factors decrease with increasing temperature for all the analytes investigated. A good linear correlation is obtained between $\ln \kappa$ and $1/T$. The slope of this plot is similar to that observed in reversed-phase chromatography[20]. No effects, specific to CTA, were found with respect to this general trend.

Considering the enantioselectivity at different temperatures, a small increase is observed for some analytes (TFAE, Tröger's base), whereas for others (spirobi-indanone, phenyldioxolanone) a slight decrease is observed. Probably the temperature increase causes some changes in the swelling state. This influences the structure of the adsorbent and thus to a certain degree the accessibility of the adsorption sites. In this way the enantioselectivity may be enhanced or reduced.

The plate height generally decreases dramatically at higher temperatures (Fig. 1), because of the increased diffusion velocity. Exceptions are those solutes where the kinetics of mass exchange is rapid even at low temperatures, *e.g.*, phenyldioxolanon. In these cases the decrease is not as dramatic, but comparable to the effects found in reversed-phase chromatography with ODS packings.

The strong influence of the temperature on all of the chromatographic parameters (κ, α, H) means that the temperature is a most important optimization parameter. The decrease in capacity factors in combination with the increase in plate number, and sometimes also in enantioselectivity, makes the use of elevated temperatures favourable in those cases where no racemization of analytes takes place at elevated temperatures. It allows reduced analysis times, increased peak heights and thus lower detection limits for the analytes.

The influence of the temperature on the chromatographic resolution may differ, as is seen in Table III. At constant enantioselectivity the effect of a decreasing capacity factor is by far overcompensated by the increase in N, for most analytes showing medium or low efficiency. In these cases (TFAE, Tröger's base) the resolution is therefore much improved with increasing temperature. Parallel to the improvement in the chromatographic resolution, the minimum analysis time is reduced significantly (Table III). With methanol–ethanol mixed mobile phases the temperature effect is less

TABLE I

STRUCTURES OF CHIRAL COMPOUNDS USED AS TEST ANALYTES

The given capacity factor values, κ_1, refer to the first enantiomer eluted and are measured with the eluent ethanol–water (96:4, v/v) at 50°C.

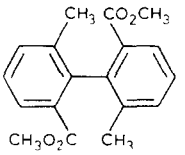
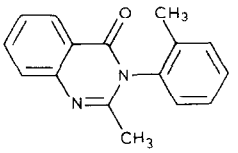
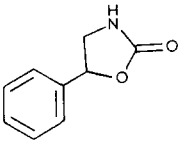
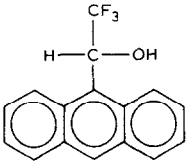
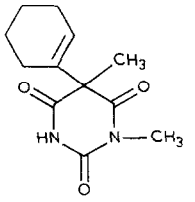
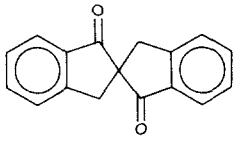
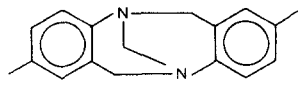
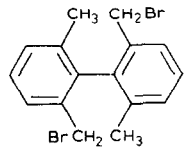
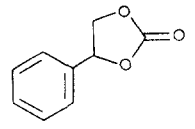
Code No.	*Solute*	κ	*Structure*
1	*o,o'*-Dimethyl-*o,o'*-di(methoxycarbonyl)biphenylene	0.26	
2	Methaqualone	0.27	
3	5-Phenyltetrahydrooxazol-2-one	0.53	
4	2,2,2-Trifluoro-1-(9-anthryl)ethanol (TFAE)	0.57	
5	Hexobarbital	0.83	
6	2,2'-Spirobiindan-1,1'-dione	0.95	
7	Tröger's base	1.05	
8	*o,o'*-Dimethyl-*o,o'*-di(bromomethyl)biphenylene	1.11	
9	4-Phenyl-1,3-dioxolan-2-one	1.84	

TABLE II

PLATE HEIGHT, H, CAPACITY FACTOR, κ, AND ENANTIOSELECTIVITY, α, FOR VARIOUS CHIRAL ANALYTES AS A FUNCTION OF THE TEMPERATURE

The code numbers of the solutes are as in Table I. The indices I and II indicate the first and second isomers eluted. Flow-rate: 1 ml/min.

Code	*Solute*	*T (°C)*	κ_I	H_I	κ_{II}	H_{II}	α
Eluent: ethanol–water (96:4, v/v)							
4	TFAE	30	0.73	690	1.94	1470	2.66
		40	0.61	520	1.68	1060	2.75
		50	0.52	320	1.45	660	2.79
		60	0.44	220	1.22	435	2.77
7	Tröger's base	30	1.33	1370	2.75	1980	2.07
		40	1.09	1180	2.32	1800	2.13
		50	0.99	800	2.12	1170	2.14
		60	0.80	445	1.77	710	2.21
Eluent: ethanol–methanol–water (76.8:20:3.2, v/v)							
4	TFAE	30	0.51	630	1.12	980	2.23
		40	0.49	610	1.08	950	2.23
		50	0.42	510	0.98	650	2.34
		60	0.38	480	0.89	560	2.38
6	Spirobiindanone	30	1.37	670	2.88	1000	2.11
		40	1.23	690	2.54	1010	2.06
		50	1.00	610	2.03	870	2.03
		60	0.86	400	1.72	840	1.99
7	Tröger's base	40	0.92	790	1.47	1160	1.60
		50	0.82	720	1.38	870	1.68
		60	0.71	385	1.24	570	1.76
9	Phenyloxolanone	40	1.98	180	3.83	170	1.94
		50	1.73	–	3.26	–	1.88
		60	1.44	110	2.68	150	1.86

pronounced than with a pure ethanolic eluent, since the addition of methanol causes an improvement in the efficiency itself (*cf.*, next section).

In cases where the enantioselectivity decreases with increasing temperature or those which show an high efficiency even at low temperatures, the resolution slightly decreases with increasing temperature (phenyldioxolanone, spirobiindanone). The positive effect with respect to the analysis time and the detection limit remains.

At temperatures above 60°C and at flow-rates > 0.42 mm/s, the long-time stability of the packing with respect to efficiency may be reduced and should be controlled. A slight increase in the plate height may result from a strong compression of the swollen bed. At 50°C and flow-rates of 0.28 mm/s ($\approx$ 1 ml/min with a column of 10 mm I.D.) no significant loss in efficiency with time was observed over a period of several months.

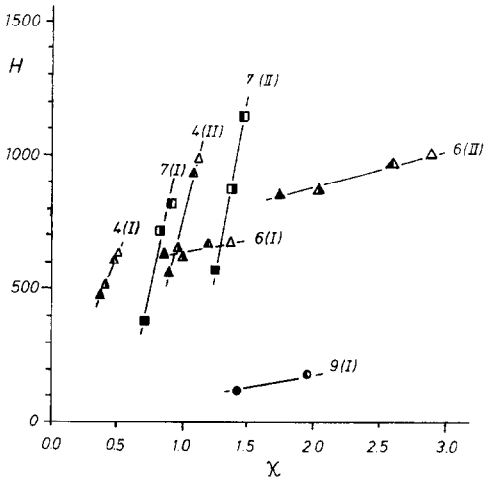


Fig. 1. Plate heights, H, and capacity factors, κ, as a function of the temperature. Code numbers of solutes as in Table I. Temperature codes: △, ▲, □ 30°C; ◭, ◐, ◧ 40°C; ◮, ◑, ◨ 50°C; ▲, ●, ■ 60°C. Eluent: methanol–ethanol–water (20:76.8:3.2, v/v); flow-rate 1 ml/min.

TABLE III

CHROMATOGRAPHIC RESOLUTION, R_S, AND ANALYSIS TIME, $t_{R,min}$, AS A FUNCTION OF COLUMN TEMPERATURE

$t_{R,min}$ = Minimum analysis time defined in eqn. 2; $t_{R(25)}$ = analysis time using a column of length 25 cm; flow-rate 1 ml/min.

		T (°C)			
		30	40	50	60
Eluent: ethanol–water (96:4, v/v)					
4 TFAE	R_S	11.4	12.6	14.8	15.8
	$t_{R,min}$	23.2	19.2	15.0	12.6
	$t_{R(25)}$	44.1	40.2	36.8	33.3
7 Tröger's base	R_S	7.6	7.8	9.2	11.5
	$t_{R,min}$	32.6	38.3	30.5	21.7
	$t_{R(25)}$	56.3	49.8	46.8	41.6
Eluent: ethanol–methanol–water (76.8:20:3.2, v/v)					
4 TFAE	R_S	7.3	7.2	8.2	8.1
	$t_{R,min}$	26.1	25.9	21.7	21.0
	$t_{R(25)}$	31.1	31.2	29.7	28.4
3 Spirobiindanone	R_S	11.2	10.2	9.6	9.9
	$t_{R,min}$	31.8	31.2	28.4	24.7
	$t_{R(25)}$	58.2	53.1	45.5	40.8
7 Tröger's base	R_S	–	4.7	5.5	7.3
	$t_{R,min}$	–	47.4	39.2	27.7
	$t_{R(25)}$	–	37.1	36.7	33.6
10 Phenyloxolanone	R_S	–	23.4	–	22.5
	$t_{R,min}$	–	18.6	–	14.7
	$t_{R(25)}$	–	72.5	–	55.2

Influence of the mobile phase composition

The eluent composition can be varied within certain limits. These limits are given on the one hand by the solubilities of the analytes in the eluent mixture and on the other hand by the solubility of the CTA material in the solvent and by the stability of its swollen state. The spectrum of the solvent components which can be used conveniently includes mainly alcohols, water, alkanes and some ethers. No ketones or chloroalkanes should be used.

This paper shows the results of an investigation of the influence of methanol, 1-propanol, 2-propanol, water and cyclohexane at various concentrations mixed with ethanol. The composition of these mixed phases is given together with their abbreviations in the Appendix. Fig. 2 shows the dependence of ln κ of several analytes on the concentration of methanol in methanol–ethanol mixed mobile phases. Fig. 3 shows the capacity factors of the second eluted enantiomers for all analytes of Table I and for some non-chiral, non-polar aromatic hydrocarbons in various mixed mobile phases. Fig. 4 shows the corresponding enantioselectivity coefficients, α, and Fig. 5 the corresponding plate height values, H.

(i) Methanol. For all solutes investigated, a decrease in the capacity factor with increasing methanol content in methanol–ethanol mixed mobile phases is observed. In most cases a fairly linear correlation of ln κ with the volume fraction of methanol is found (Fig. 2). The slopes of such plots are sometimes different for different compounds, reflecting differences in the molecular sizes and in the adsorption mechanisms, as discussed above.

It is noteworthy that the addition of methanol causes a decrease in the capacity factors not only for polar but also for non-polar solutes (Fig. 3b). With swcrCTA adsorbents, the elution power of an eluent component is determined not only by the strength of the competitive interaction with the binding sites and by the strength of interaction with the solute, but also by its steric size. Methanol is a stronger competitor than ethanol for the adsorption of the solutes at narrow adsorption sites. Secondly, the addition of methanol changes the swelling state of the adsorbent and in this way the number of adsorption sites available. Both these effects overcompensate by far the

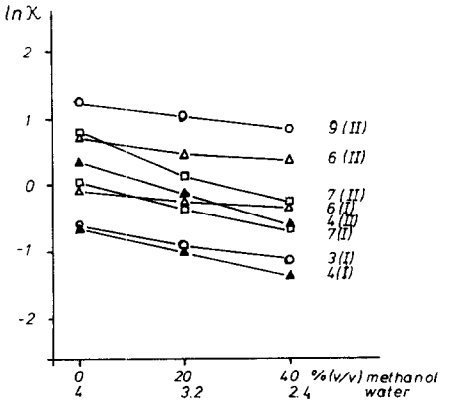


Fig. 2. Dependences of the logarithm of the capacity factors of various analytes on the volume fraction of methanol in methanol–ethanol–water mixed mobile phases. Code numbers of solutes as in Table I. Chromatographic conditions: flow-rate 1 ml/min; temperature 50°C.

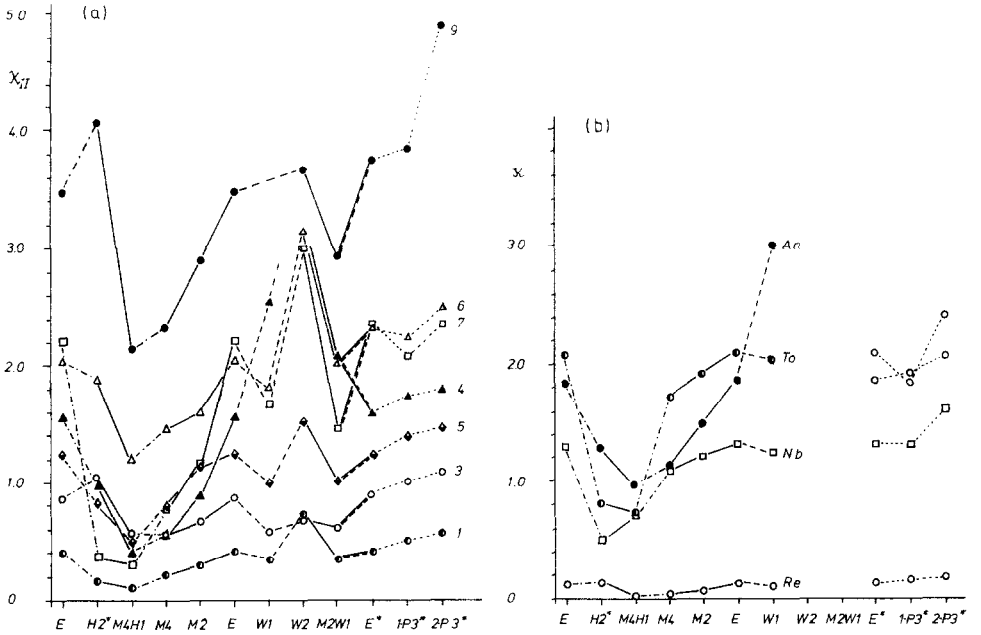


Fig. 3. Capacity factors of the second enatiomers eluted of various analytes obtained at various mobile phase compositions. (a) Compounds listed in Table I. Code numbers of solutes as in Table I. (b) Non-chiral, non-polar aromatic hydrocarbons: An = anthracene; To = toluene; Nb = nitrobenzene; Re = resorcinol. Solid lines: methanol content changed. Dotted lines: propanol content changed. Broken lines: water content changed. — · — · —, Cyclohexane content changed. Eluent codes as in the Appendix. Chromatographic conditions: Flow-rate 1 ml/min; temperature 50°C. The asterisks indicate a lower pressure drop, as specified in Table IV.

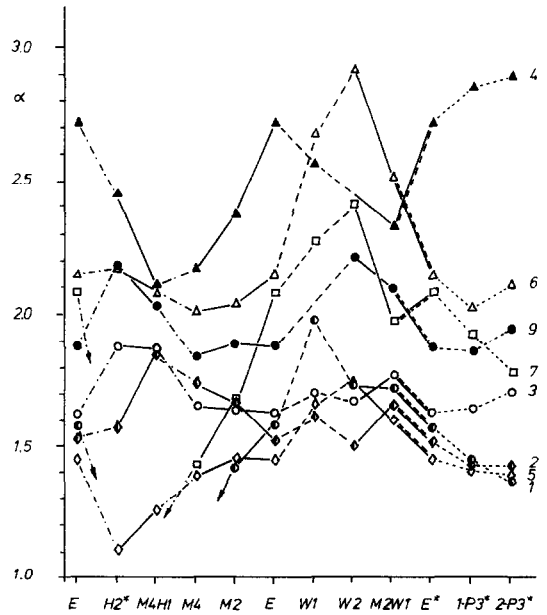


Fig. 4. Stereoselectivity, α, at various compositions of the mobile phases. Symbols and chromatographic conditions as in Fig. 3. Eluent codes are given in the Appendix.

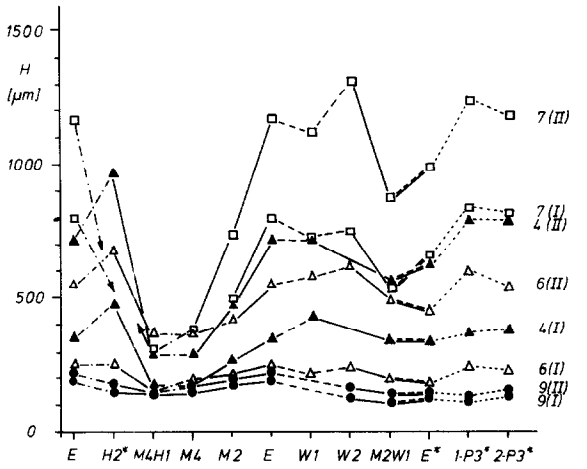


Fig. 5. Plate height, H, at various compositions of the mobile phase. Symbols and chromatographic conditions as in Fig. 3. Eluent codes as in the Appendix.

increase in the mobile phase activity coefficients of non-polar solutes (resulting from the lower solvation power of methanol for these types of analytes in comparison to ethanol), which, alone, would cause an increase in the capacity factors.

The enantioselectivity generally decreases upon addition of methanol (Fig. 4). This can be understood by assuming methanol to be a competitor especially for the narrow sites, which are most important for chiral recognition, and, further, that in the altered swelling state the number of narrow sites available is smaller.

Of great consequence is the strong decrease in plate height upon the addition of methanol (Fig. 5). This effect is found for all substances with exception of those compounds (phenyldioxolanone, phenyloxazolone) where low plate height values were found even before. Obviously, the reduced importance of the narrow sites, where the adsorption kinetics is slow, causes changes in the same direction as a reduction in viscosity.

The addition of methanol offers similar advantages as does the elevation of temperature: shorter analysis time, improved detection limit and little altered, or only slightly reduced, resolution. This is illustrated in Table IV, which shows that the decrease in the chromatographic resolution is small, since the gain in efficiency balances the loss in enantioselectivity in most cases.

(ii) Cyclohexane. In most cases, the addition of cyclohexane to the eluent leads to a decrase in retention. The magnitude of this effect is dependent on the analyte structure. It results either from an enhanced competitive adsorption of this solvent component at non-polar adsorption sites, and/or from the improved solvation power of the mixed solvent for non-polar analytes (reduced mobile phase activity coefficient). It should be mentioned in this context that methanol as well as cyclohexane are stronger displacers than ethanol on swcrCTA materials.

With the addition of cyclohexane an improvement in stereoselectivity is often observed. (Exceptions are TFAE and hexobarbital, where a slight decrease in α is observed, and Tröger's base, which cannot be resolved in systems with cyclohexane.) The influence of cyclohexane on the plate height is rather small. The increased plate

TABLE IV

CHROMATOGRAPHIC RESOLUTION, R_S, AND MINIMUM ANALYSIS TIME, $t_{R,min}$, AS A FUNCTION OF THE SOLVENT COMPOSITION

Flow-rate: 1 ml/min. Temperature: 50°C. Eluent code as given in the Appendix.

Code	*Solute*		*Eluent*					
			E	*M2*	*M4*	*M4H1*	*W1*	
Back pressure with pure ethanol: 90–95 atm[a]								
9	Phenyldioxolanone	R_S	20.0	20.7	18.6	20.9	–	
		$t_{R,min}$	20.2	16.9	16.1	13.5	–	
		$t_{R(25)}$	67.1	58.4	49.8	47.0	–	
3	Phenyloxazolone	R_S	7.6	7.4	7.6	8.6	6.9	
		$t_{R,min}$	22.0	20.2	18.5	16.1	20.4	
		$t_{R(25)}$	27.9	24.9	23.3	23.0	23.6	
6	Spirobiindanone	R_S	15.0	13.8	13.5	13.1	18.8	
		$t_{R,min}$	18.2	16.8	16.5	15.0	13.4	
		$t_{R(25)}$	45.5	38.9	36.9	32.9	42.0	
4	TFAE	R_S	14.4	10.1	8.0	6.1	6.7	
		$t_{R,min}$	16.0	16.8	17.4	20.8	19.1	
		$t_{R(25)}$	38.3	28.2	23.3	21.0	53.1	
7	Tröger's base	R_S	9.0	5.6	3.8	–	9.1	
		$t_{R,min}$	32.1	34.4	41.7	–	26.4	
		$t_{R(25)}$	48.0	32.3	26.6	–	40.1	
5	Hexobarbital	R_S	–	5.5	4.6	–	6.4	
		$t_{R,min}$	–	34.6	35.2	–	28.0	
		$t_{R(25)}$	–	31.8	26.9	–	29.9	
			E	*1-P3*	*2-P3*	*H2*	*W2*	*M2W1*
Back pressure with pure ethanol about 30 atm[a]								
9	Phenyldioxolanone	R_S	25.0	26.5	27.5	30.1	31.1	28.6
		$t_{R,min}$	17.1	16.4	19.3	15.2	13.5	12.3
		$t_{R(25)}$	71.3	72.5	88.4	75.9	69.9	58.8
3	Phenyloxazolone	R_S	9.4	10.4	12.2	11.8	–	9.0
		$t_{R,min}$	17.9	17.3	15.3	15.5	–	16.1
		$t_{R(25)}$	28.2	29.9	31.1	30.5	–	24.0
6	Spirobiindanone	R_S	17.2	14.4	16.7	14.2	26.8	19.9
		$t_{R,min}$	16.3	20.1	18.8	18.3	13.9	13.6
		$t_{R(25)}$	46.5	48.3	52.4	43.1	62.1	45.2
4	TFAE	R_S	15.1	15.6	16.0	8.2	–	15.0
		$t_{R,min}$	15.5	15.7	15.7	21.7	–	18.4
		$t_{R(25)}$	39.0	40.9	41.7	29.6	–	46.1
7	Tröger's base	R_S	10.6	7.5	7.1	–	12.6	7.9
		$t_{R,min}$	28.6	36.7	42.3	–	28.4	27.8
		$t_{R(25)}$	50.4	45.9	50.1	–	59.9	36.5
5	Hexobarbital	R_S	–	5.0	4.9	–	7.0	6.6
		$t_{R,min}$	–	43.3	45.3	–	32.5	27.1
		$t_{R(25)}$	–	35.9	36.8	–	37.6	29.9

[a] A small influence of the back pressure on the capacity factors, the enantioselectivities and the plate heights is observed[19]. The significance of the differences in the back pressure values given can be judged by comparing the data sets obtained with the eluent E.

heights in several cases probably result from the limited access of cyclohexane molecules to narrow "slow"-type adsorption sites.

The increase in enantioselectivity connected with a decrease in retention is of great practical importance for reducing the analysis time and improving resolution (Table IV).

(iii) 1-Propanol and 2-propanol. The addition of propanol induces a moderate increase in the capacity factors of all analytes investigated. This effect is especially pronounced for rather small analytes with polar structures, *e.g.*, phenyldioxolanone). It seems to be quite the opposite of the effect observed with methanol. 2-Propanol generally shows a stronger effect than 1-propanol.

The enantioselectivity is little influenced by the addition of propanol within the range of 0–30% (v/v).

The plate height generally increases (opposite effect with methanol). At least partially, this effect is caused by an increase in the viscosity of the eluent.

Generally, the addition of propanol does not offer substantial advantages. In special cases the slight increase in selectivity might be of importance.

(iv) Water. The influence of water strongly depends on its concentration in ethanol and on the solute structure. At low concentrations [10% (v/v) water added] the solute capacity factors decrease in most cases. As with methanol, this effect may be due to changes in the swelling state of CTA and to a competitive effect at the narrow adsorption sites (which are probably important for the more polar interactions). Unlike methanol, however, water increases the enantioselectivity considerably.

At higher concentrations of water [20% (v/v) added] the retention increases in many cases. This effect depends on the analyte structure and is expected owing to the increase in the mobile phase activity coefficient. It is especially pronounced for analytes with large non-polar parts.

That both effects, reduction of the available free adsorption sites as well as the increase in activity coefficients, are operative is demonstrated by phenyldioxolanone, where the capacity factor of the first enantiomer eluted decreases, whereas that of the second increases.

Surprisingly, the plate height is little influenced by the addition of water in the concentration ranges described and for the analytes investigated. This may be due to a compensation of viscosity effects and displacement effects from the narrow adsorption sites.

The increase in enantioselectivity is the most important potential of water in the optimization of separations on CTA.

(v) Ternary mixtures. A useful combination of advantageous effects may be obtained by using ternary mobile phases of the type ethanol–methanol–cyclohexane and ethanol–methanol–water. In both these solvents the advantages of dramatically reduced capacity factors and significantly reduced band broadening (by methanol) can be combined in most cases with an enhanced enantioselectivity (by water or cyclohexane). This allows one to influence the analysis time, resolution and detection limit in the desired direction (*cf.*, Table IV).

The chromatograms of the racemic analytes Tröger's base, hexobarbital and TFAE in Fig. 6 illustrate the influence of the solvent composition on the chromatographic resolution and the analysis times.

When interest is not restricted only to chiral separations, CTA adsorbents offer

an high potential for tuning the selectivity also between different compounds by rather small changes in the mobile phase composition, as is seen from Fig. 3.

Optimum flow velocity and optimum column length

The plate height contribution which results from the mass exchange in the packed bed, H_b (*cf.*, refs. 21 and 22), is found to be the predominant contribution to the total plate height, H_{tot}[19,20]. H_b is known to increase approximately linearly with the flow velocity[21–25]. Because of the predominance of this linear contribution of H_b one also observes an approximately linear increase in the total plate height, H_{tot}, with u at flow velocities higher than u_{min} (Fig. 7). The high contribution of H_b causes the minimum of H to be situated at very low flow velocities, lower than 0.139 mm/s. (This value corresponds to 0.5 ml/min in a column with 10 mm I.D. and to about 0.09 ml/min in a column with 4 mm I.D) Fig. 7 illustrates the dependence of H on the flow velocity for analytes with different steric structures. The slope is not correlated with the capacity factor, as usual, but with the structure of the analyte, as has been pointed out in the first section and in ref. 19. Fig. 7 demonstrates the high potential of improving the separation by applying low flow velocities. However, its use results in very long analysis times.

In the range above u_{min} the total plate height, H_{tot}, can be expressed in most cases to a good approximation by

$$H_{tot} = a_i u \tag{3}$$

where a_i is the structure- and solvent-dependent slope. Such a simple linear dependence of H on u has interesting implications. Assuming N_{min} to be the minimum plate number

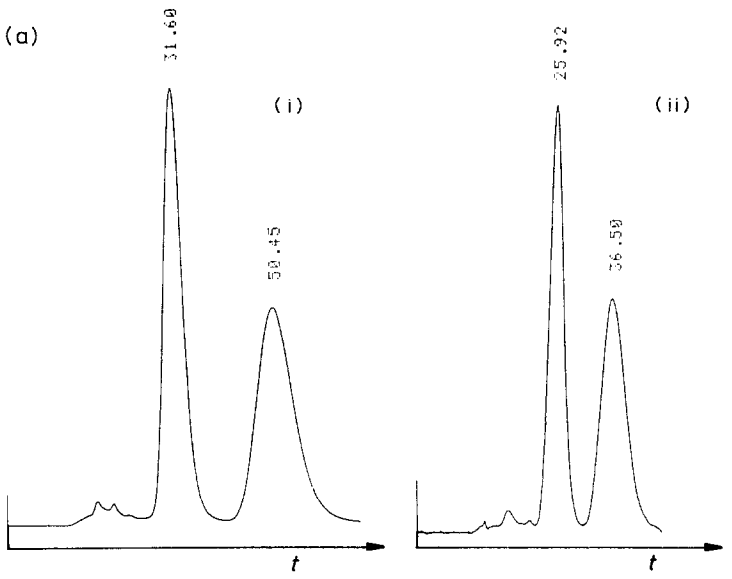


Fig. 6.

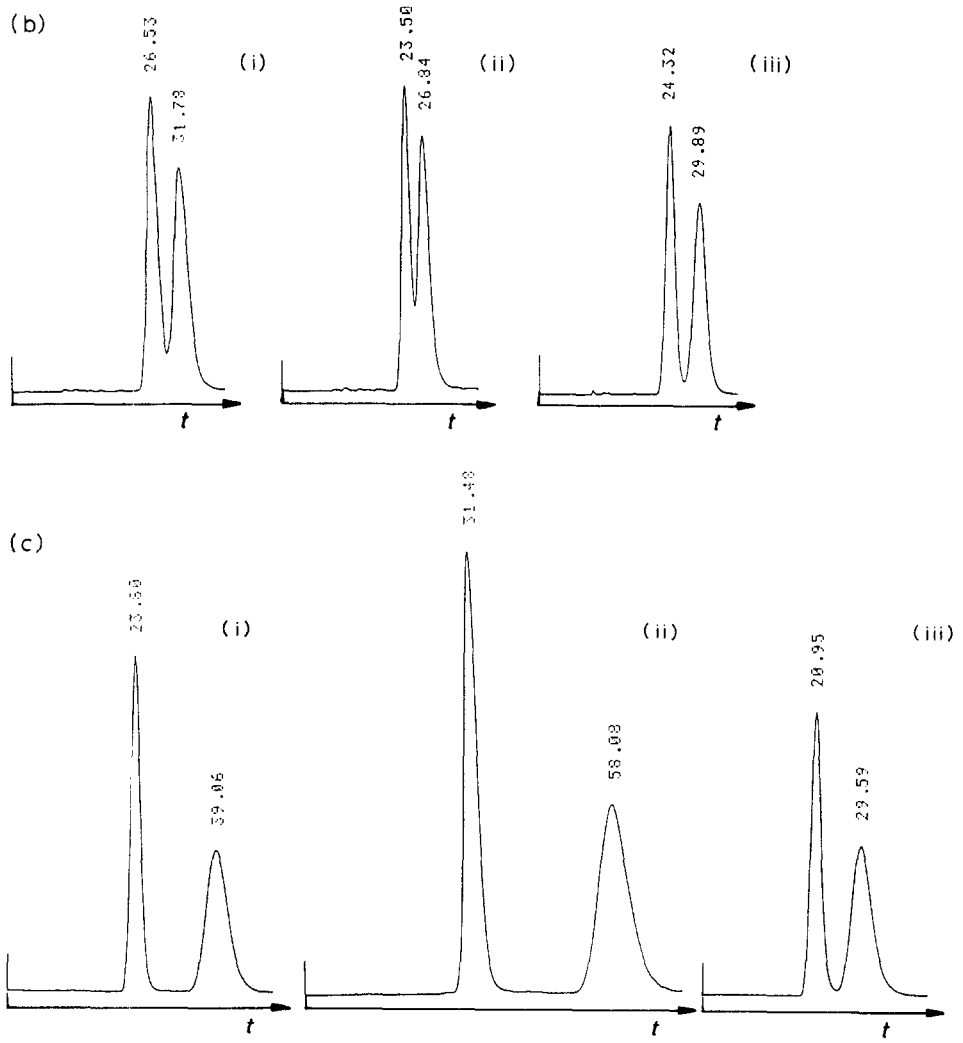


Fig. 6. Chromatograms of optical isomers as a function of the solvent composition. (a) Tröger's base: (i) ethanol–water (96:4) (E); (ii) methanol–ethanol–water (20:67.2:12.8) (M2W1). (b) Hexobarbital: (i) methanol–ethanol–water (20:76.8:3.2) (M2); (ii) methanol–ethanol–water (40:57.6:2.4) (M4); (iii) methanol–ethanol–water (20:67.2:12.8) (M2W1). (c) TFAE: (i) ethanol–water (96:4) (E); (ii) ethanol–water 86.4:13.6) (W1); (iii) cyclohexane–ethanol–water (20:76.8:3.2) (H2). Retention times in minutes. Flow-rate: 1 ml/min. Temperature: 50°C. Injection volume: 20 μl.

needed for a desired resolution in a given chiral separation problem, the minimum column length, L_{min}, which allows to obtain $\bar{N}_{i,min}$ at a certain value of $\bar{H}_i$, is given by eqn. 4:

$$L_{min} = \bar{N}_{i,min}\, \bar{a}_i u \tag{4}$$

If one is able to adjust the column length to L_{min}, *e.g.*, by using small combinable columns one obtains for the minimum analysis time:

$$t_{Ri,min} = \bar{N}_{i,min}\, \bar{a}_i\, (1 + \kappa_{i,II}) \tag{5}$$

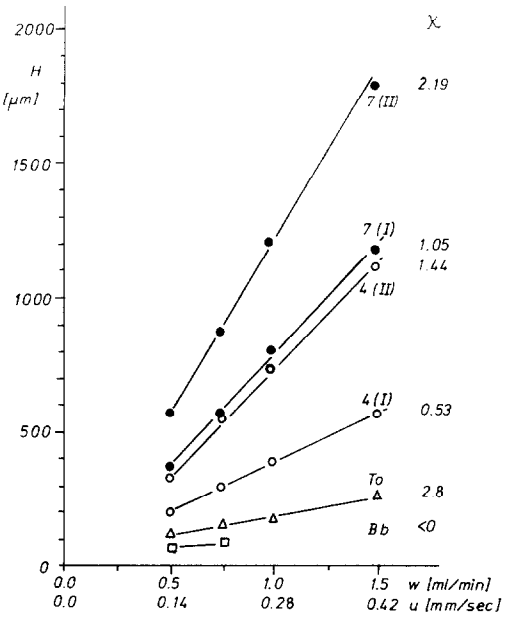


Fig. 7. Plate height as a function of the linear flow velocity, u. Code numbers of solutes as in Table I; Bb = tri-*tert.*-butylbenzene; To = toluene. Eluent: ethanol–water (96:4, v/v). Temperature: 50°C.

In this case, and unlike the usual chromatographic situation, the analysis time is no longer a function of the flow velocity, but only of $\bar{N}_{i,\min}$ and $\bar{a}_i$, the mean value of the H/u slopes of the two enantiomers. This may be an interesting aspect for highly repetitive analyses or in preparative separations, where an adjustment of the column length to the given problem might be worthwhile.

Column loadability

The loadability of swcrCTA packed columns is generally high. With concentration overloading, previous investigations[19] showed that the peak symmetry, plate height and elution time of the peak maximum is not significantly influenced up to an injected mass of 10–20 μg in a 250 mm × 10 mm column, with 20-μl injection volumes. The corresponding concentration is near the solubility limit for several substances.

Very recently, results differing from these have been reported for other types of analytes (substituted phenylthiazolinethiones)[26]. There, a slight tailing and a slight shift of the elution time of the peak maximum has been observed. Since this effect was found to differ for two enantiomers, or even to be in opposite directions, the enantioselectivity was found to be considerably influenced by the loading. In such a case the quality of a separation under concentration overloading cannot be predicted on the basis of the data obtained with low concentrations.

However, the injectable mass and the throughput can be considerably increased (further) by using volume overloading. To calculate the maximum allowable injection volume without loss in resolution, it is assumed that the chromatographic resolution is little affected (<5%) up to an injection volume equivalent to σ_{vi}, the standard

TABLE V

CALCULATED VALUES FOR THE MAXIMUM ALLOWABLE INJECTION VOLUME, V_{inj}max (5%), WHICH AFFECTS THE CHROMATOGRAPHIC RESOLUTION OF ENANTIOMERS BY NOT MORE THAN 5% IN A swcrCTA COLUMN (250 mm × 10 mm I.D.) AND APPROXIMATE MAXIMUM ALLOWABLE INJECTION VOLUME, V_{inj} max (t.b.), AT WHICH THE FIRST PEAK ELUTED JUST TOUCHES THE SECOND ONE

Temperature: 50°C. Eluent: ethanol–water (96:4, v/v). Pressure drop: 70 atm.

Code	*Solute*	V_{inj} *max(5%) (μl)*	V_{inj} *max(t.b.) (ml)*
9	Phenyldioxolanone	1170	≈15
3	Phenyloxazolone	600	≈0.6
6	Spirobiindanone	920	≈7
4	TFAE	880	≈5.2
7	Tröger's base	1740	≈2

deviation of the analyte peak in volume units. This maximum allowable injection volume depends on type and structure of the analyte and on the temperature and the solvent composition, as was pointed out in the discussion of the efficiency in CTA columns. Table V shows these maximum allowable injection volumes for a 25 cm × 1 cm I.D. column.

In preparative scale separations, however, volume overloading is usually extended until the peaks are just touching. The maximum allowable injection volume in this sense depends on the resolution which can be obtained under the conditions chosen. For non-tailing peaks, these injection volumes are evaluated approximately by extrapolation and are given in Table V.

Pressure stability

The pressure stability of the column tested was excellent, especially after cleaning the bottom frit after the first 2.5 l of eluent had passed through the column at elevated temperature. At a temperature of 50°C and a flow velocity of 0.28 mm/s (1 ml/min), the back pressure, enantioselectivity, plate height and peak symmetry were constant and reproducible. It is important that changes in the solvent composition had no significant additional influence on the increase in the back pressure and plate height after reverting to the original solvent. Obviously, the changes in the swelling state were fully reversible.

CONCLUSIONS

The adsorption mechanism and the mass exchange process in swollen crystalline CTA packings are found to be different from those usually observed with silica and alkylsilica adsorbents. The plate heights are strongly dependent on the structures of the analytes but not on their capacity factors. The influence of temperature and solvent composition on the plate height, stereoselectivity and retention are often quite dissimilar to those usually observed.

The following general trends are found within the range of temperature or solvent composition investigated. Enhanced temperature induces improved efficiency, reduced retention and slightly increased or decreased stereoselectivity. Addition of

methanol, propanol, water and cyclohexane as solvent components to ethanol results predominantly in the following effects: (i) methanol [up to 40% (v/v)], improved efficiency, reduced retention and reduced stereoselectivity; (ii) cyclohexane [10 and 20% (v/v)], improved stereoselectivity, reduced retention and approximately constant or slightly reduced efficiency; (iii) propanol [up to 30% (v/v)], in some cases enhanced stereoselectivity, increased retention and reduced efficiency; (iv) water [up to 20% (v/v)], strongly enhanced stereoselectivity, approximately constant efficiency and reduced or enhanced retention, depending on the water concentration and the hydrophobicity of the solutes. The addition of two mobile phase modifiers allow one to combine the advantages of the single solvent components: (v) methanol–cyclohexane or methanol–water yield enhanced stereoselectivity, improved efficiency and reduced retention.

A reduction of the flow velocity strongly improves the efficiency. This effect is observed down to u_{min}, which lies at very low flow velocities of about 0.14 mm/s. The approximate linear dependence of the plate heights on the flow velocity means that the minimum analysis time is independent of the flow velocity.

The minimum analysis time is determined by the minimum column length needed to obtain sufficient resolution in a given chromatographic separation problem. It is determined therefore by the stereoselectivity and by the capacity factor of the second eluted enantiomers and the mean plate number for a given pair of enantiomers. Tables III and IV contain also the analysis time for columns of a constant length of 25 cm. This analysis time depends only on the capacity factors of the analytes. These data are useful for realistic cases where the column length cannot be varied and when the resolution obtained is sufficient.

From the point of view of chromatographic practice, it is important that a significant reduction of the analysis time can be achieved in most cases by increasing the temperature and by adding methanol, cyclohexane and/or water. This procedure allows in addition a significant lowering of the detection limit by improving the efficiency and by reducing the capacity factors of the analytes.

The stability of the pressure and column efficiency was good with the column investigated. Owing to the low plate height contribution arising from the dispersion in the streaming part of the mobile phase, the column has to be judged as a well packed high-performance column.

APPENDIX

Composition (%, v/v) of mixed mobile phases and their abbreviations:

E	Ethanol–water (96:4)
H2	Cyclohexane–ethanol–water (20:76.8:3.2)
M4H1	Methanol–cyclohexane–ethanol–water (40:10:48:2)
M4	Methanol–ethanol–water (40:57.6:2.4)
M2	Methanol–ethanol–water (20:76.8:3.2)
W1	Ethanol–water (86.4:13.6)
W2	Ethanol–water (76.8:23.2)
M2W1	Methanol–ethanol–water (20:67.2:12.8)
1P3	1-Propanol–ethanol–water (30:67.2:2.8)
2P3	2-Propanol–ethanol–water (30:67.2:2.8)

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