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Cholesteryl-silica stationary phase for liquid chromatography Comparative study of retention behavior and selectivity

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Abstract

Retention mechanism, separation possibilities and physicochemical properties of a laboratory-synthesized packing material for high-performance liquid chromatography (HPLC) containing cholesterol moiety immobilized on silica matrix were studied. In order to exhibit the original properties of the new phase, two referential materials – an octadecylsilica and an acylaminopropylsilica phase – were prepared starting from the same silica adsorbent. The chromatographic behavior of the three columns was examined with special regard to retention and selectivity in the reversed-phase HPLC mode employing a variety of mobile phase compositions. Special attention has been paid to possible specific applications of the phases and to temperature effects on retention and selectivity of selected polycyclic aromatic hydrocarbons and other analytes of highly diversified structure.

Keywords: Stationary phases, LC; Retention factors; Selectivity; Cholesterol-silica stationary phases; Polynuclear aromatic hydrocarbons; Alkylbenzenes

1. Introduction

Chemically bonded phases are widely adapted for many different analytical purposes, mainly for high-performance liquid chromatography (HPLC), solid-phase extraction (SPE), membrane dropping and filtration [1–3]. Numerous silica-based stationary phases have been introduced into the market. However, there is still a great demand for universal packing material which can be utilized in routine analysis as well as for columns suited to selected specific applications. Packings with hydrophobic alkyl chains of different lengths are frequently used in various environmental, pharmacological or biochemical investigations. Separation of complex analytes of differentiating chemical nature poses some problems when utilizing those conventional stationary phases. Such analysis requires packing with specific structural properties. For example, the materials containing terminal and/or internal polar groups built into the alkyl chains [4].

The separation efficiency in reversed-phase HPLC is mostly influenced by the binary hydro–organic mobile phase composition together with the under-surface properties of the packing material [5]. The most progress in increasing efficiency of specific separations has been achieved due to introduction of a variety of column types, e.g., cyclodextrins [6],

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Pirkle's phases [7] or aryl phases [8], as well as the materials containing mixed ligands [9] or exhibiting liquid-crystalline properties [10,11]. Pidgeon and Venkatarum [12] elaborated an original phase of the so-called immobilized artificial membrane (IAM) type that was successfully applied for amino acid and peptide separations. Kaliszan and co-workers [13–17] took advantage of this phase for basic drug separations and for predicting their biological activity. Similarly, specific separation properties are also possessed by the so-called pseudo-membrane aminopropyl (AP) packing, containing apart from the residual silanols, *N*-acylamino groups built into the long hydrophobic chains. Owing to such a structure, a solvolytic cover is created on the surface of the silica matrix [4,5,15,18].

A good example of a phase conforming to specific requirements of the separated compounds, e.g., polycyclic aromatic hydrocarbons (PAHs) or chemicals of basic/acidic nature is the phase described by Jinno et al. [18]. That phase probably shows some liquid crystalline behavior. In this packing, the cholesterol moiety is bonded to a silica adsorbent via an undecenoate ligand. Such a solution opens new analytical possibilities due to the higher mobility of bonded cholesterol. Hence, the separation of drugs with more elongated structures becomes feasible. On the other hand, on account of the reproducibility, a very interesting phase is reported by Delaurent et al. [19]. However, the lack of detailed description of the separation mechanism does not permit a more precise indication of the suitability of the material.

As an extension of our previous investigations concerning the special retention properties of cholesterol bonded to a silica matrix, a series of chemically bonded phases, including cholesteric packing have been synthesized, using the same starting adsorbent (Kromasil 100, 5 μm). All steps of the preparation procedure were controlled by means of advanced physicochemical techniques, such as porosimetry, elemental analysis, ^{29}Si and ^{13}C solid-state nuclear magnetic resonance (NMR) and Fourier transform (FT) IR. Individual stationary phases (''Chol'', AP and C_{18}) showed differentiated separative properties and sometimes exhibited the pseudo-membrane (PM) properties. These properties were assessed by studying the effect of temperature (Van 't Hoff plots) and mobile phase composition on chromatographic behavior of selected compounds on the newly synthesized phases. In order to elucidate molecular mechanism of retention, the quantitative structure–retention relationship (QSRR) [13–17] approach was applied.

2. Experimental

2.1. Materials

The solid support of all phases was Kromasil 100 AT 0112 (Akzo Nobel, Bohus, Sweden). Physicochemical characteristics of the bare adsorbent are given in Table 1.

The following reagents were used for the chemical modification of the silica support material: octadecyltrichlorosilane purchased from Petrarch System (Levittown, PA, USA); γ-aminopropyltriethoxysilane and triethylamine (Fluka, Buchs, Switzerland); palmityl chloride (E. Merck, Darmstadt, Germany); morpholine (Riedel-de Haën, Seelze, Germany). Cholesteryl chloroformate 98% was purchased from Sigma–Aldrich (Gillingham, UK). Organic solvents were of HPLC-grade (J.T. Baker, Deventer, Netherlands). The test solutes used were of various origins. Water was taken from a Milli-Q RG system (Millipore Intertech, Bedford, MA, USA).

2.2. Apparatus

The porosity parameters such as specific surface area, pore volume and pore diameter of bare Kromasil 100 were determined by the low-temperature nitrogen adsorption–desorption method using a

Table 1
Physico–chemical characteristics of bare Kromasil 100 (AT 0112) (Eka Nobel, Sweden) used as a support of chemically bonded phases

Characteristics	Abbreviation	Unit	Value
Mean particle size	d_p	μm	5
Particle shape	–	–	Spherical
Specific surface area	S_{BET}	m^2/g	310
Pore volume	V_p	ml/g	0.82
Mean pore diameter	D	nm	10
Concentration of OH groups	α_{OH}	$\mu mol/m^2$	7.1
Trace amounts of metals	C_M	ppm	29

Model 1800 Sorptomatic instrument (Carlo Elba, Milan, Italy). For the inductively coupled plasma (ICP) atomic emission spectroscopy (AES) studies a Model PU-7000 apparatus with an ultrasonic nebulizer type U-5000AT (Unicam, Cambridge, UK) was applied to determine the metal impurities concentration (C_M) in the adsorbent. Respective data are collected in Table 1.

The degree of coverage of silica support with bonded ligands (parameters α_{RP} and N) was calculated from carbon (P_C) and nitrogen (P_N) contents determined by an elemental analysis with a CHN analyzer Model 240 (Perkin-Elmer, Norwalk, CT, USA) (Table 2). The values of surface coverage with the bonded groups were calculated by the formula reported previously [4,5].

The ^{13}C and ^{29}Si solid-state NMR experiments were performed on an MSL 300 spectrometer (Bruker, Rheinstetten, Germany) in the magic-angle spinning (MAS) module. The FT-IR spectra were recorded on a Spectrum 2000 machine (Perkin-Elmer). The details of both measurements have been given elsewhere [4,9,15,20].

The phases under study were packed into 100 mm×4.6 mm I.D. stainless steel columns. All columns were packed using a DT 122 packing pump (Haskel, Burbank, CA, USA) under the pressure of 50 MPa. Technical details of the procedure are described in our earlier papers [9,15,20].

The test solutes for QSRR analysis were subjected to molecular modeling by the HyperChem package with the extension ChemPlus (HyperCube, Waterloo, Canada). Calculations employing the Statgraphics Plus-6.0 package (Manugistic, Rockville, MD, USA) were run on a personal computer.

Chromatographic measurements were made using a HP-1050 liquid chromatograph system (Hewlett-Packard, Waldbronn, Germany) equipped with a diode array detector and a Vectra QS/HP computer with the ChemStation-2 software for data collection and control of the process. The columns were thermostatted in a Julabo F25 cryostat (Julabo Labortechnic, Seelbach, Germany).

2.3. Synthesis and column packing procedure

Chemical surface modification of bare Kromasil was carried out under vacuum in a glass reactor without reagent contact with the external environment.

The silica gel was heated to 140°C under vacuum (10^{-2} Pa) for 8 h in a specially designed glass reactor. Then, the temperature was decreased to 120°C and reaction mixtures were added: (A) γ-aminopropyltriethoxysilane for product A (''Chol'' phase); (B) γ-aminopropyltriethoxysilane for product B (AP phase) and (C) octadecyltrichlorosilane+ morpholine for product C (C_{18} phase).

After 12 h, the reaction products (A, B, C) were washed with toluene, methanol and hexane. The synthesis products A and B were placed in a glass reactor and heated to 100°C. Next, product A was treated with triethylamine in the presence of dried toluene. Then, a cholesteryl carbamate with morpholine as a reaction activator was added. Product B was modified with palmitoyl chloride with morpholine. The reactions were carried out during 12 h, and final products (A and B) were washed as described above.

Slurry was prepared from the modified silica and

Table 2
Surface characterization of modified silica[a]

Packing	Percentage		Coverage	
	P_C (%)	P_N (%)	α_{RP} (μmol/m^2)	N (number of groups/nm^2)
SG-^{polymeric}NH$_2$	4.47	1.33	4.67	2.80
SG-^{polymeric}AP	15.19	1.20	2.83	1.69
SG-^{polymeric} Chol	21.195	1.21	2.64	1.58
SG-^{polymeric} C_{18}	19.02	–	3.74	2.24

[a] Where: SG-^{polymeric}NH$_2$ = silica gel modified with aminopropylsilane; SG-^{polymeric}AP = silica gel modified with aminopropylsilane and palmitoyl acid chloride; SG-^{polymeric} CHOL = silica gel modified with cholesteryl moieties; SG-^{polymeric} C_{18} = silica gel C_{18} phase; P_C = percentage value of carbon; P_N = percentage value of nitrogen; α_{RP}, N = surface coverage; N (number of groups/nm^2) = $0.6\alpha_{RP}$ (μmol/m^2) [29].

2-propanol and placed in a sample container of the column packing apparatus. The columns were packed under a pressure of 50 MPa using methanol as delivery solvent. The preparation procedure, reaction mechanism and synthesis conditions are reported elsewhere [9,15,20].

3. Results and discussion

3.1. Surface characterization of stationary phase materials

Table 1 shows the physicochemical characteristics of bare silica gel. All the parameters are closely similar to those expected for the ''theoretically optimal'' adsorbent [4]. It is worth mentioning here that according to our previous investigations, Kromasil characterizes very good reproducibility of important chromatographic parameters.

Successful completion of the bonding reaction has been first illustrated by the FT-IR spectra of the starting adsorbent (A), an intermediate (B) and the final product (C) (Fig. 1). At a frequency of $\nu \approx 3400$ cm^{-1} the reduction of intensity of signals attributed to residual silanols on the modified silica spectra (B and C) takes place in relation to bare adsorbent (A). The cholesteric phase displays the most prominent peak at $\nu = 2961$ cm^{-1} which is characteristic of a bonded hydrocarbon and a readily identifiable peak at $\nu = 1697$ cm^{-1} which is due to the carbonyl functions. The characteristic peak of the aminopropylsilica spectrum at $\nu = 1635$ cm^{-1} is attributed to the bending vibrations of amino groups. That peak almost disappeared from the ''Chol'' phase spectrum. Bending vibrations of methyl and methylene groups are responsible for the signals at $\nu = 1470$ cm^{-1}. These signals indicate considerable hydrophobicity of the surface covered by an organic stationary phase.

Fig. 2 displays the ^{13}C cross polarization (CP) MAS-NMR spectra of bonded cholesteric phase. The carbon atoms of cholesteric moiety attributed to observed signal have been marked. Creating of the undersurface structure of a polymer type has been clearly confirmed by the lack of characteristic signal at $\delta = -2.5$ ppm [20]. Interpretation of ^{13}C CP-MAS-NMR spectrum has been accomplished according to

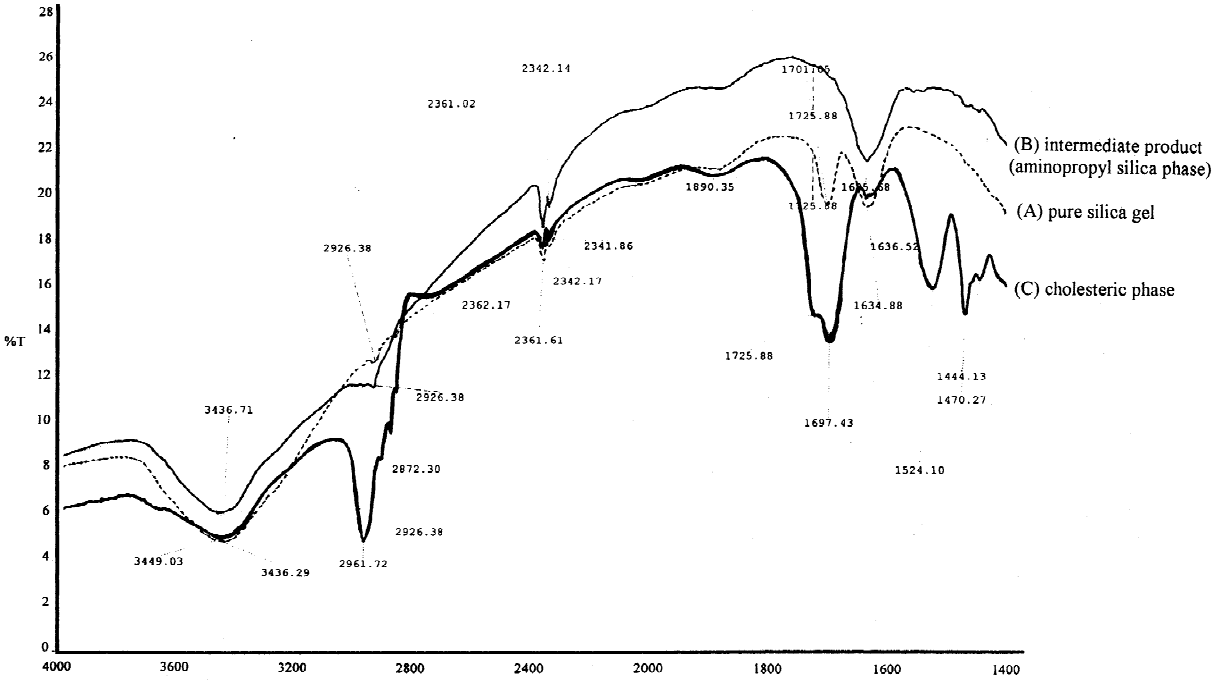

Fig. 1. FT-IR spectra of starting adsorbent (A), intermediate (B) and final product (C) of the ''Chol'' phase.

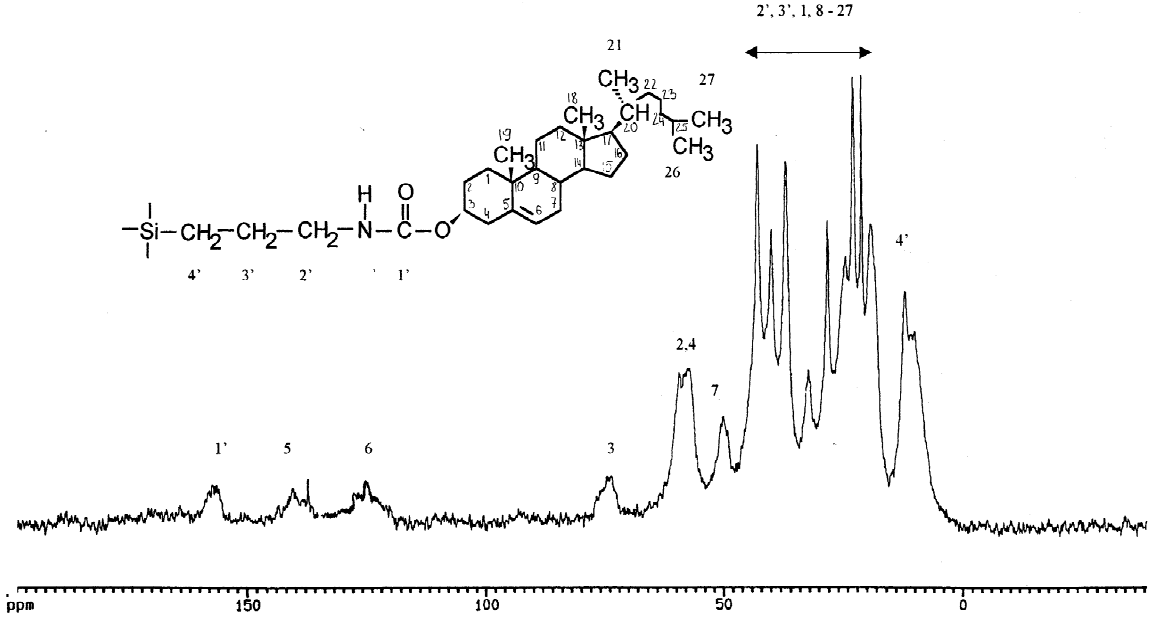

Fig. 2. ^{13}C CP-MAS-NMR spectra of the bonded cholesteric packing.

the rules presented previously [9,15,20]. Possible structures ascribed to the packings studied are presented in Fig. 3. The probable undersurface structure of the ''Chol'' phase is given in Fig. 3A. In this case, as well as for the AP material (Fig. 3B), the groundwork was bonding the aminopropyl group. From the values of surface coverage density listed in Table 2, a dense, homogenic undersurface layer of organic stationary phase can be expected. Along with the density of bonded groups, Table 2 contains also the carbon and nitrogen contents from elemental analysis. These quantities indicate the hydrophobic nature of the prepared packings. However, analytical characteristics of the undersurface layer structure can eventually be obtained from chromatography.

3.2. Chemometric analysis of retention parameters of a predesigned series of test analytes

Retention properties of the ''Chol'' phase were examined employing the QSRR method [13–17]. The aim was to elucidate the molecular mechanism of chromatographic retention as well as to predict separation behavior of individual substances.

Generally, a very good linear correlation of log k' vs. mobile phase composition has been observed. That allowed normalization of the retention parameter to the log k'_w values obtained by extrapolation of the linear relationships. Based on the log k'_w data from chromatographic experiments and structural parameters [16,17] of a series of 24 test analytes several QSRR equations were derived.

A multiple regression equation relating the retention parameters to the structural parameters based on the linear solvation energy relationships (LSERs) is as follows:

$$\begin{aligned}\log k'_w = {} & 0.4550(\pm 0.4041) \\ & - 0.7481(\pm 0.2935)\alpha_2^H \\ & - 3.1141(\pm 0.3095)\beta_2^H \\ & + 3.3121(\pm 0.3313)V_x \end{aligned} \quad (1)$$

where: α_2^H is the effective hydrogen bond acidity, β_2^H the effective hydrogen bond basicity and V_x the characteristic volume of McGowan based on the complexation scale of Abraham et al. [16]. Correlation between the observed experimentally and the calculated by Eq. 1 log k'_w data is illustrated in Fig. 4.

The QSRR equation employing structural parameters from molecular modeling of analytes is as follows:

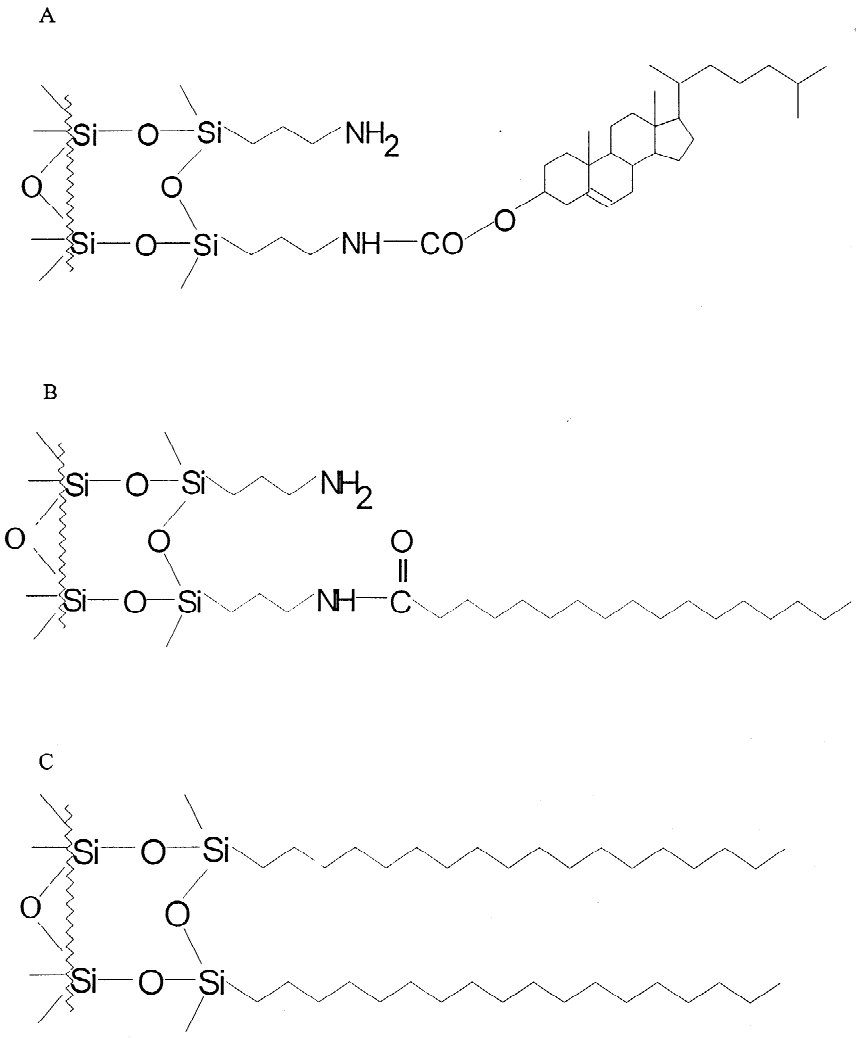

Fig. 3. Possible structures of the stationary phase materials: (A) ''Chol'', (B) AP and (C) C_{18}.

$$\log k'_w = -0.9856(\pm 0.6817) + 5.3280(\pm 1.4095)\mathrm{CHARGE}_{MIN} + 0.0180(\pm 0.0020)\mathrm{SAS} - 0.1079(\pm 0.0262)\mu^2 \quad (2)$$

where: CHARGE_{MIN} is the maximum atomic electron excess, SAS the molecular surface area accessible for water and μ^2 the square of total dipole moment. Correlation between the observed experimentally, and the calculated by Eq. 2 $\log k'_w$ data is illustrated in Fig. 5.

Detailed discussion of QSRR equation characteristics for the AP, C_{18} and IAM phases have been presented previously [14–17]. Here we wish only to note that QSRR Eqs. 1 and 2 confirm specific, distinctive retention properties of the ''Chol'' phase indicating at the same its highest similarity to typical octadecylsilica C_{18} phase.

3.3. Hydrophobicity and silanol activity determination

To observe the difference in chromatographic

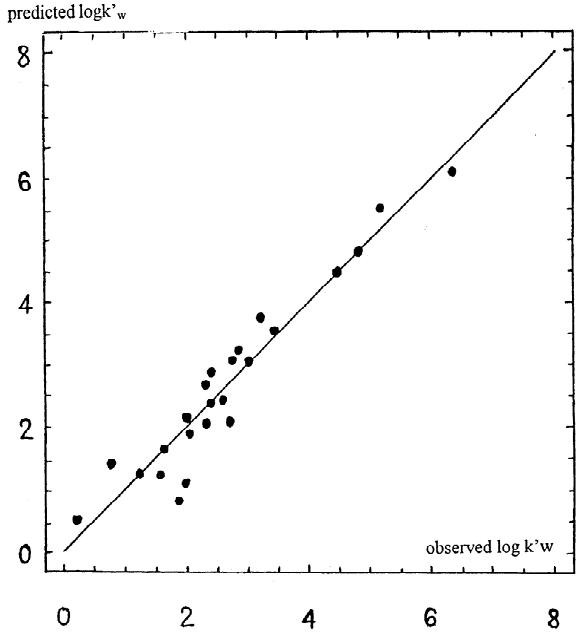

Fig. 4. Correlation between logarithms of retention factor extrapolated to pure water, log k'_w, of a series of test analytes observed experimentally and calculated from Eq. 1.

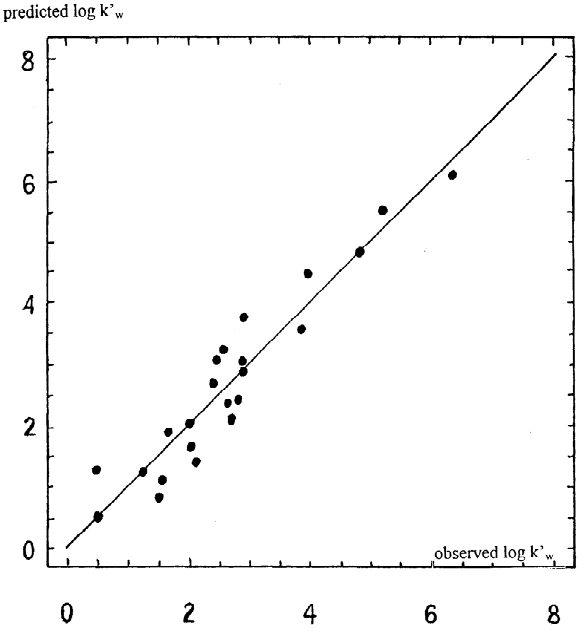

Fig. 5. Correlation between logarithms of retention factor extrapolated to pure water, log k'_w, of a series of test analytes observed experimentally and calculated from Eq. 2.

properties of prepared materials, simple hydrophobicity and silanol activity investigations have been undertaken.

The hydrophobicity parameter was calculated according to Engelhardt and Jungheim [21]. Toluene and ethylbenzene in a mobile phase of methanol–water (80:20) were used to monitor the hydrophobic properties. Calculated values for all columns were contained in a 1.4–1.48 range. The highest value was calculated for the C_{18} column, which also showed the highest surface coverage.

The amount of silanols reflecting hydrogen bonding capacity has been calculated according to Tanaka et al. [22] with caffeine and phenol as test samples in methanol–water (30:70). Once more, the highest relation as in case of hydrophobicity of caffeine to phenol was obtained for C_{18} packing. It was three-times greater than for the AP phase. Because of the complicated structure of bonded ligands in ''Chol'' phase and in spite of the lowest coverage density, $\alpha_{\text{caffeine/phenol}}$ are greater for the ''Chol'' phase than for AP material. All values mentioned above are listed in Table 3.

3.4. Comparative chromatographic analysis

Fig. 6 displays the chromatograms of PAHs obtained on the three columns considered. It is evident that resolution obtained on the ''Chol'' column is good enough in comparison to the separation obtained with the C_{18} packing. Retention times are comparable and in a few cases the ''Chol'' column shows better selectivity towards PAHs. It is particularly evident in the case of pairs of analytes: benzo[*a*]pyrene and chrysene (peaks 9 and 10 resolution) and indeno[1,2,3-*cd*]pyrene and benzo[*ghi*]perylene (separation of peaks 15 and 16). Unfortunately, the separation of benzo[*b*]fluoran-

Table 3
Hydrophobicity and silanol activity

Packing	Hydrophobicity[a]	Silanol activity[b]
Chol	1.43	0.995
AP	1.40	0.370
C_{18}	1.48	1.133

[a] Hydrophobicity calculated according to Ref. [21].
[b] Silanol activity calculated according to Ref. [22].

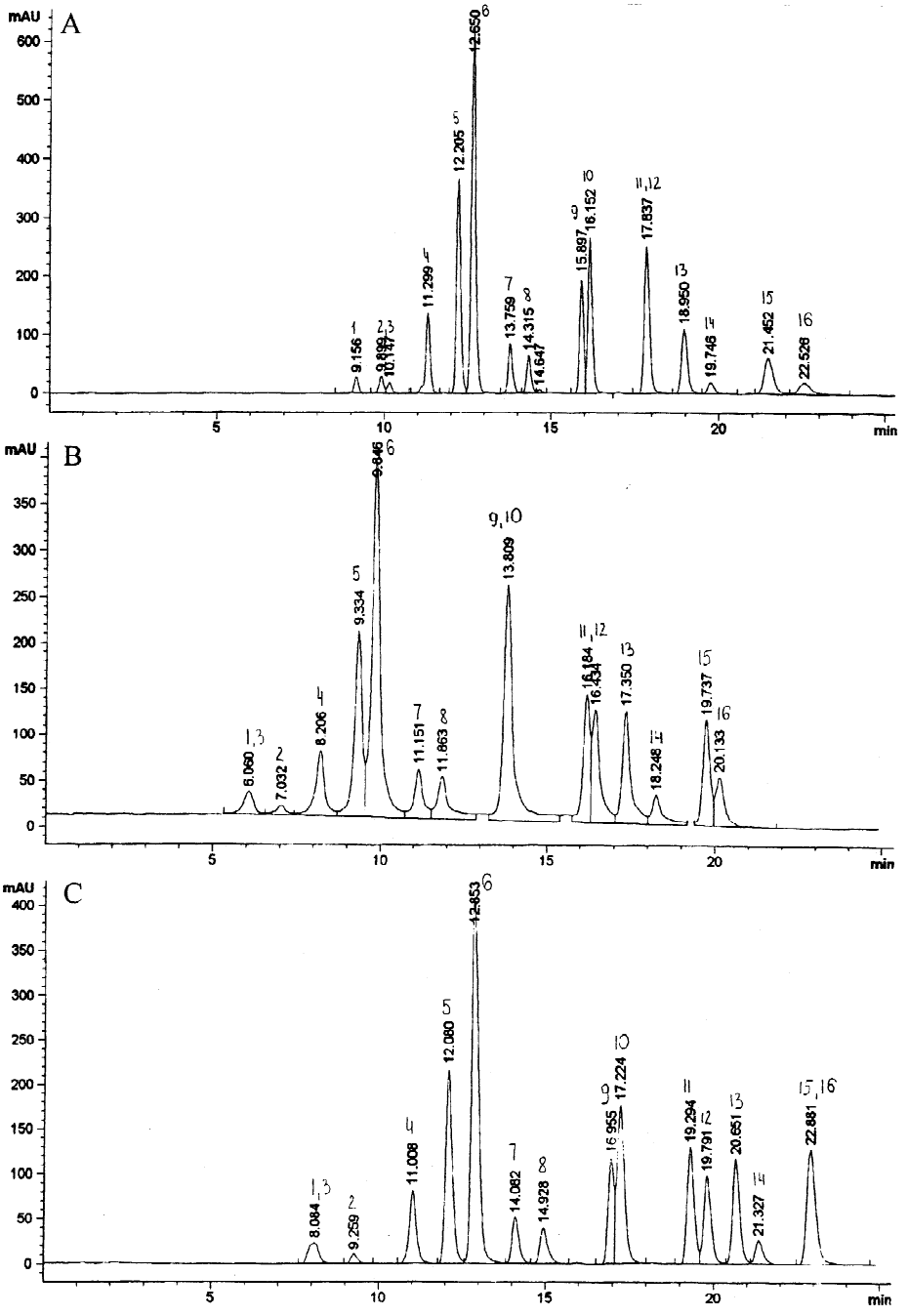

Fig. 6. Chromatograms of 16 PAHs determined on the columns under study: (A) ''Chol'', (B) AP and (C) C_{18}. Analytes: 1=naphtalene, 2=acenaphtalene, 3=acenaphtene, 4=fluorene, 5=phenantrene, 6=anthracene, 7=fluoranthene, 8=pyrene, 9=benzo[*a*]anthracene 10=chrysene, 11=benzo[*b*]fluoranthene, 12=benzo[*k*]fluoranthene, 13=benzo[*a*]pyrene, 14=dibenz[*a,h*]anthracene, 15=indeno[1,2,3-*cd*]pyrene, 16=benzo[*ghi*]perylene. Mobile phase: 50% ACN to 100% ACN in 5 min, hold at 100% of ACN in 25 min, λ=254 nm, flow-rate: 1 ml/min.

thene from benzo[*k*]fluoranthene is not achieved on the ''Chol'' column.

Fig. 7 shows separation factors (k') of compounds of different chemical nature (basic, acidic and neutral) for the three columns under study. For almost all cases (except toluene) the k' parameters are highest for the ''Chol'' column and they are close to those for the C_{18} phase. This indicates the similar mechanism of retention processes on the ''Chol'' and the C_{18} packings. That similarity is not preserved only for a compound with an acidic functional group (nitrobenzoic acid). This observation suggests special behavior of the ''Chol'' column as regards acidic substances.

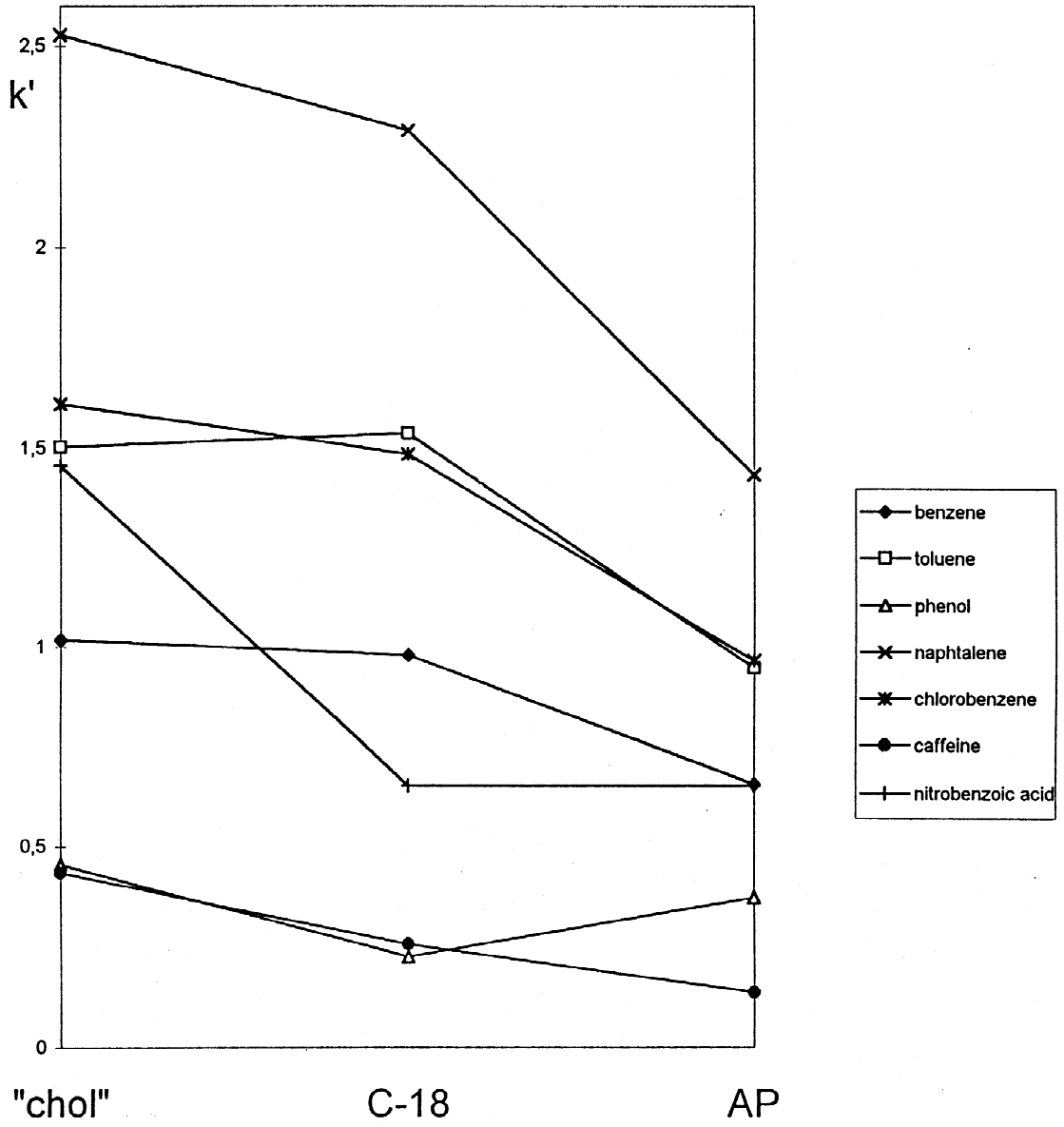

Fig. 7. Retention factor (k') of compounds of differentiated chemical nature for the columns studied. Mobile phase MeOH–water (80:20); $\lambda = 254$ nm, flow-rate: 1 ml/min.

A similar kind of relationship can be observed in Fig. 8 which shows the correlation between the number of carbon atoms connected to benzene ring of the analyte and the logarithm of retention factor of five alkylbenzenes determined with the methanol–water (80:20). The slope for the ''Chol'' phase has a value close to that for the alkylamide AP column, although the intercepts are much more different than for the ''Chol'' and the C_{18} phases. Similar slopes observed for the ''Chol'' and AP packings are probably the result of similar retention mechanism on both columns with respect to small molecules of analytes possessing π-electrons.

The type of stationary phase material studied has no influence on the selectivity factors (α) listed in Table 4. All the values of α parameter indicate a very good separation of test analytes on the three examined materials.

3.5. Temperature effects on retention

Systematic investigations of temperature effects on ordering direction of octadecyl ligands have been carried out for some time [23–25]. Kasturi et al. [26] studied the reordering/resolvating problem in the case of alkylamide phases. A non-linear thermal behavior (deviation from Van 't Hoff plots) has been described and explained. Earlier experiments done by Pesek and co-workers [27,28] suggested that varying temperature and mobile phase composition could help to demonstrate the similarities and differences between the packings with possible liquid crystal behavior. This was also confirmed in our studies.

The three considered packings, among them the ''Chol'' column, have been examined at temperatures ranging from 298 to 348 K (25–75°C) using an isocratic mobile phase of acetonitrile–water (60:40). Fig. 9 contains Van 't Hoff plots for benzene, naphthalene and anthracene for ''Chol'' (A), AP (B) and C_{18} (C) columns. For the ''Chol'' column one can expect two points of line breaking reflecting the phase transition of the aminopropyl group (marked phase transition) and cholesteryl moiety. This can also be observed in the case of the alkylamide phase. Values of $\log k'$ corresponding to given $1/T$ are much higher for ''Chol'' than for the AP phase. This fact, expressing a greater mobility and more effective screening of the surface by AP ligands has also a confirmation in the results of analysis of under-surface structure. It especially concerns steric spacers between individual chains: the long hydrocarbon spacers in the case of AP phase and the stericly enlarged molecules of ''Chol'' phase. Differences between these two packings regarding the temperature effects on retention result from opposite screening mode of the residual silanols and aminopropyl chains, which remain on silica surface after the modification processes. Studies of temperature effects on retention indicate some degree of ordering of ligands on the support surface. The liquid crystal transition temperatures for bonded cholesteric as well as behavior of AP and C_{18} phases in varied temperature range could be determined by differential scanning calorimetry (DSC). With regard to differentiated coverage density of aminopropyl ligands in relationship to both cholesteryl and alkylamide ligands, in preliminary chromatographic measurements non-linear behavior of those firsts is much more visible. Preliminary measurements confirmed that non-linear chromatographic experiments were useful in characterizing conformational changes of bonded phases, including the new type of packing. That topic will be developed in our further studies.

4. Conclusions

By chemical modification of silica adsorbent surface by means of trifunctional modifiers, a stationary phase for HPLC containing cholesterol moiety as well as a hydrocarbonsilica C_{18} and an alkylamide phase with high and controlled coverage density were obtained. The cholesteric stationary phase ''Chol'' meets the demands of a modern packing for liquid chromatography in a reversed-phase mode. Despite of modest plate efficiency (ca. 23 000 theoretical plates/m), the selectivity and resolution provided by the new phase are sufficient. Retention processes taking place on the ''Chol'' phase are well described by the QSRR equations in terms of the LSER-based and molecular modeling-calculated structural descriptors of test analytes. Structural features formed by cholesterol ligand on the surface of silica create a kind of pseudo-membrane layer. The ''Chol'' phase can be useful in

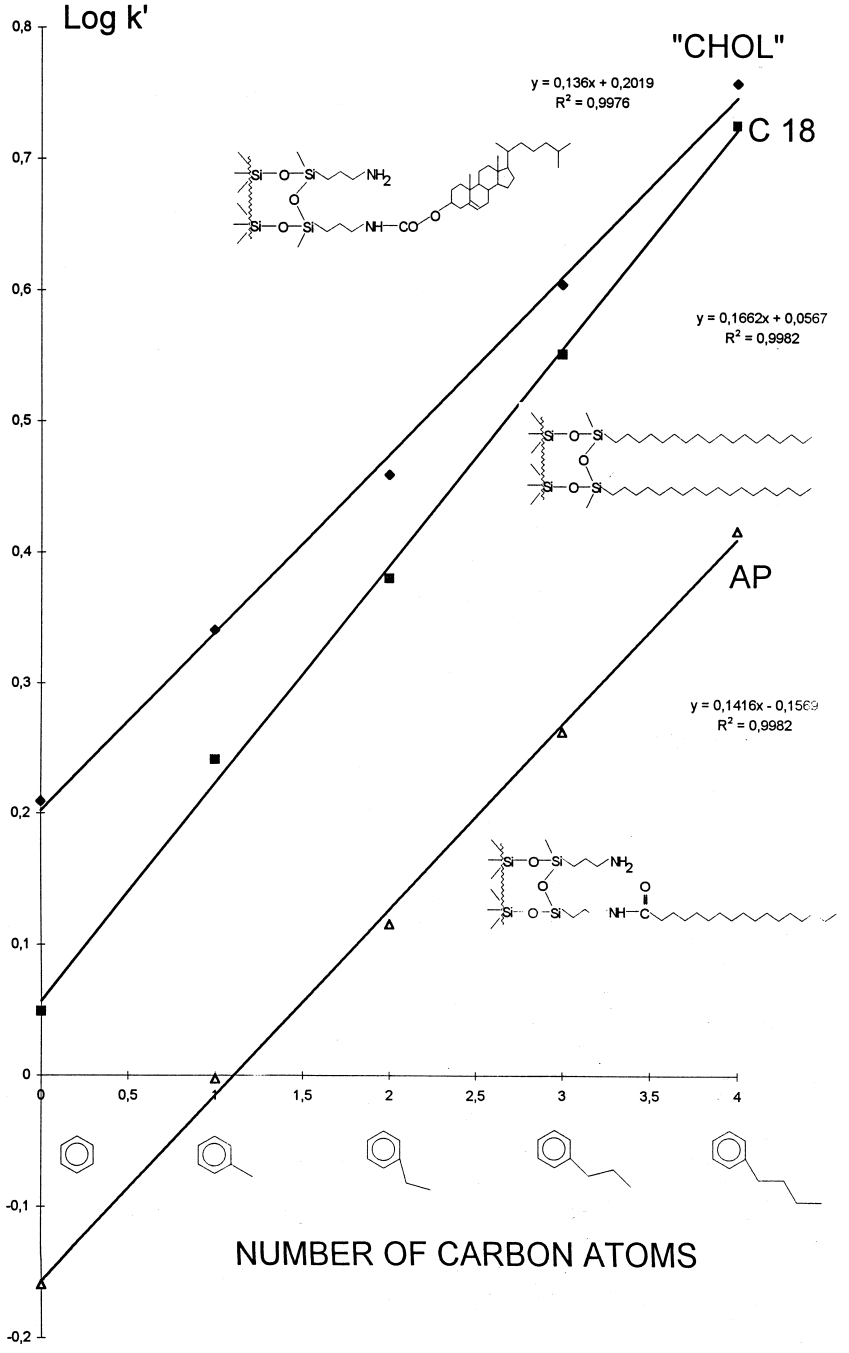

Fig. 8. Correlation between the number of carbon atoms connected to benzene ring and the logarithm of retention factor for five alkylbenzenes. Mobile phase MeOH–water (80:20); $\lambda = 254$ nm, flow-rate: 1 ml/min.

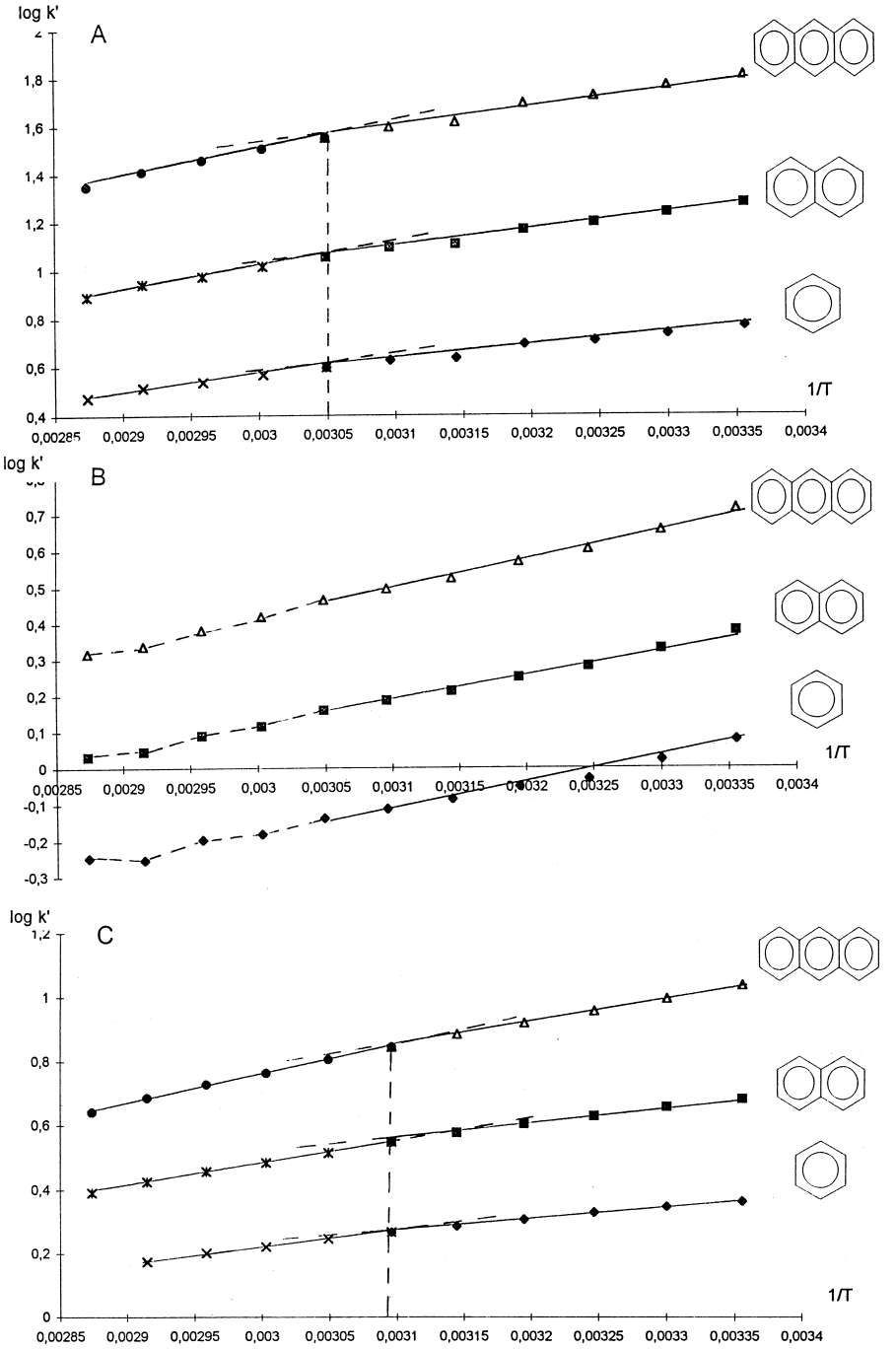

Fig. 9. Exemplary Van 't Hoff plots for ''Chol'' (A), AP (B) and C_{18} (C) phases. Mobile phase ACN–water (60:40); $\lambda = 254$ nm, flow-rate: 1 ml/min.

Table 4
Selectivity for alkylbenzenes [mobile phase: MeOH–water (80:20)]

$\alpha_{toluene/benzene}$			$\alpha_{ethylbenzene/toluene}$			$\alpha_{propylbenzene/ethylbenzene}$			$\alpha_{butylbenzene/propylbenzene}$		
CHOL	AP	C_{18}	CHOL	AP	C_{18}	CHOL	AP	C_{18}	CHOL	AP	C_{18}
1.35	1.43	1.55	1.31	1.31	1.38	1.43	1.40	1.48	1.42	1.42	1.49

pharmacokinetic studies, especially in modeling the penetration of xenobiotics through biological membranes. The column is also suitable for work at a wide temperature range without loosing its efficiency and resolution.

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