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Gas-phase synthesis, properties and some applications of acylamide stationary phases for high-performance liquid chromatography

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ABSTRACT

Acylamide stationary phases for high-performance liquid chromatography were synthesized by a successive gas-phase modification of silica gel with γ-aminopropyltriethoxysilane and benzoyl chloride, benzoic or stearic acid. Derivatization of amino phases with carboxylic acids at temperatures above 150°C requires no activation of carboxyl groups or the use of condensing reagents. The stationary phases produced were studied by IR spectrometry. The formation of amide groups on the aminopropylsilica surface was confirmed and the presence of stable ester-type surface compounds with silanol groups was detected. The effect of the pH of the eluent on the retention of nucleic acid components on acylamide stationary phases was investigated. Examples of the chromatographic separation of nucleosides, amino acid enantiomers and oligomers of N-(2,3-epoxypropyl)carbazole are presented.

INTRODUCTION

Chemically bonded silica stationary phases (SPs) for high-performance liquid chromatography (HPLC) are at present prepared by modification of silica gel with organosilicon or other compounds in a solvent. However, there is an alternative synthetic approach. Wikström *et al.* [1] and Nawrocki and Aue [2] described a gas-phase method for the silanization of SPs for HPLC, consisting in treatment of silica gel with organosilicon compound vapours at reduced pressure. This, owing to the possibility of carrying out the modification at elevated temperatures, considerably shortens the reaction time, which results in a dense monolayer of modified coating, and also requires no organic solvents. Comparison of the chromatographic properties of the prepared materials with those of SPs synthesized by standard methods demonstrated the former to have improved characteristics (high efficiency and good symmetry of peaks).

Hence the capabilities of the gas-phase modification of SPs for HPLC deserve a thorough study. From the cited papers [1,2] it is clear that this method can yield high-quality organosilicon coatings. However, the synthesis of many SPs also includes subsequent stages such as the bonding of molecules of various organic modifiers and derivatives of carboxylic acids [3–5], amino acids [6–8], etc., to active functional groups (amino, halo and epoxyalkyl) of immobilized silanes. In contrast to silanizing reagents,

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many of these compounds at ordinary (and sometimes also at elevated) temperatures are in a solid (crystalline) state, which at first glance would seem to preclude their use in the gas-phase method for the derivatization of SPs. However, under a moderate vacuum (of the order of 10^{-1} Torr) and at elevated temperatures (150–300°C), even such almost non-volatile substances as amino acids and nucleic acid bases can sublimate without decomposition [9–11]. We used this property previously in a gas-phase modification of highly dispersed pyrogenic silicas with some amino acids [12] and oxopyrimidines [13]. It was ascertained [12] that the carboxyl groups of amino acids react relatively readily with aminoalkyl groups of silica under vacuum at temperatures above 150°C with the formation of the corresponding surface amides, without the need for condensing reagents. This approach can apparently be employed also for other carboxylic acids, especially monofunctional types.

In this work we employed the gas-phase method for the successive modification of silica gel with γ-aminopropyltriethoxysilane and benzoyl chloride, benzoic or stearic acid and examined the IR spectra and chromatographic properties of the SPs produced for examples of separations of nucleic acid components, enantiomers of amino acids and oligomers of N-(2,3-epoxypropyl)carbazole.

EXPERIMENTAL

Materials and equipment

Materials and compounds used were Silasorb 600 silica gel (5 μm, specific surface area 550 m^2/g), adenine, uracil, cytosine and guanine (Chemapol, Prague, Czechoslovakia), L- and D-amino acids, adenosine, uridine, cytidine, guanosine and sodium salts of the corresponding 5′-monophosphates (Reanal, Budapest, Hungary). IR spectra were recorded on a UR-20 spectrophotometer (Karl Zeiss, Jena, G.D.R.) in the range 1400–1900 cm^{-1} for silica gels in the form of a suspension in Nujol and for highly dispersed pyrogenic silica in the form of thin compacted tablets. IR spectra of tablets under vacuum were measured with the use of a cell described elsewhere [14].

Stainless-steel columns (64 × 2 mm I.D.) were packed with SPs by a suspension method (200 bar, isopropanol; HPP-4001 pump, Laboratorní, Přístroje, Prague, Czechoslovakia). A column of Separon C_{18} reversed-phase sorbent (Chemapol, Prague, Czechoslovakia) was commercially available (Nauchpribor, Orel, U.S.S.R.). Chromatographic analyses were performed on Milikhrom 1A microcolumn chromatograph (Nauchpribor) with a UV detector (operating range 190–360 nm). The dynamic modification of reversed-phase sorbents with N-octyl-L-proline was carried out by a procedure similar to those described elsewhere [15,16].

Gas-phase modification of SPs

The conditions for the modification of silica gel with γ-aminopropyltriethoxysilane were similar to those described by Wikström *et al.* [1]. However, we used a reactor of the simplest design possible (Fig. 1). Silica gel was placed on the bottom of the reactor and evacuated for 1 h at 150°C. After cooling to room temperature, the reactor was disconnected from the vacuum system and a tube containing γ-aminopropyltriethoxysilane was placed in it (Fig. 1, stage I). After evacuation at room temperature the stopcock was closed and the bottom zone of the reactor, containing the reagents, was heated for 1 h at 150–170°C (II). Next, the excess of silane and

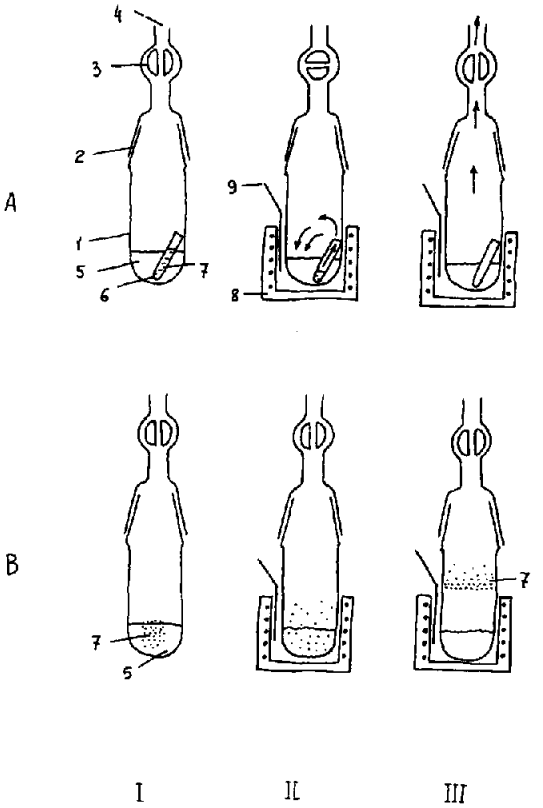

Fig. 1. Scheme of the reactor and sequence of silica gel derivatization with (A) liquid and (B) solid reagents. 1 = Reactor; 2 – joint; 3 = stopcock; 4 – to vacuum pump; 5 = silica gel; 6 = tube; 7 = reagent; 8 = oven; 9 = thermocouple. Steps: (I) preliminary pumping; (II) derivatization; (III) removal of excess of reagent.

volatile reaction products were removed by evacuation at the modification temperature (III). Derivatization of the amino phase obtained with the aid of benzoyl chloride was performed in a similar manner. The treatment was carried out at 170°C for 0.5 h (Fig. 2).

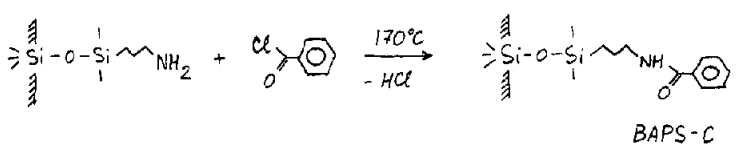

Fig. 2. Gas-phase derivatization of aminopropylsilica (APS) with benzoyl chloride.

The sequence of steps in the modification of aminopropylsilica gel (APS) with solid reagents (benzoic and stearic acids) was the same as above, but the reagents were introduced into the reactor by mixing them with silica gel (Fig. 1). In stage II, evacuation of the reactor was continued. Derivatization (Fig. 3) was conducted at

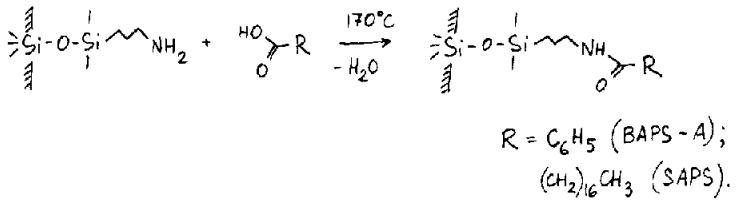

Fig. 3. Gas-phase derivatization of APS with benzoic and stearic acids.

170° C for 0.5 h. After the end of the reaction the excess of the solid reagent condensed in the cold zone of the reactor.

The chemically modified SPs produced were characterized by elemental analysis data (Table I) and IR spectra (in Nujol, Fig. 4). Before packing the columns, residual silanol groups were blocked by treatment with hexamethyldisilazane in toluene (80°C, 1 h).

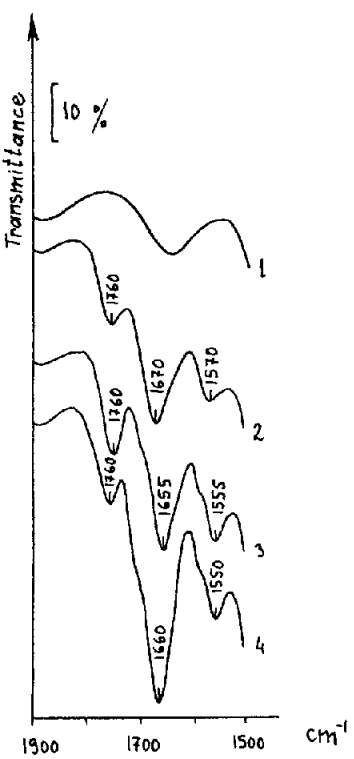

Fig. 4. Infrared spectra of (1) APS, (2) SAPS, (3) BAPS-A and (4) BAPS-C in Nujol.

RESULTS AND DISCUSSION

Modification procedure

As mentioned above, a reactor of the simplest design (Fig. 1) can be used for the gas-phase modification of silica gel. The bottom of the reactor should be as wide as possible so that the sorbent will lie as a thin layer, because highly dispersed silicas (including silica gels with particle size of the order of 10 μm) tend to "boil up" on evacuation, particularly with concurrent heating. A low volatility of stearic and benzoic acids at ordinary temperatures and the impossibility of removing the excess of the reagents from the reactor do not prevent pure materials, *i.e.*, containing no remaining modifier, from being obtained. The excess acid condenses in the reactor cold zone as a dense crust and does not contaminate silica gel when it is unloaded from the reactor. The time needed for APS acylation was less than 0.5 h in all instances (after 0.5 h the acylation yield does not increase with further treatment).

IR spectra of SPs and structure of modifying coating

The formation of amide groups on the APS surface was confirmed by the IR spectrometry. The IR spectra (Fig. 4) for the three acylation products (BAPS-C, BAPS-A and SAPS) exhibit absorption bands typical of secondary amides: amide I ($\nu_{C=O}$) at 1655–1670 cm^{-1} and amide II (δ_{NH}) at 1550–1570 cm^{-1}. Hence carboxylic acids can be employed for the acylation of amino phases without the use of condensing reagents or activation of carboxyl groups. At the same time, the presence in the IR spectra of absorption bands in the region of 1760 cm^{-1} was unexpected. This absorption also relates to $\nu_{C=O}$, but is characteristic of esters of carboxylic acids or chemisorption products, formed on condensation of carboxyl (or acid chloride) groups with silanol groups on the silica surface [17] according to Fig. 5.

Fig. 5. Formation of ester-type products on chemisorption of carboxylic acids and acyl halides on hydroxylated silica surface.

The presence of residual silanol groups on a silanized surface is not surprising. In our opinion, it is striking that, despite the extreme hydrolytic unstability of ≡SiOCO– bonds [17], bands at 1760 cm^{-1} are observed in the IR spectra of acylamide SPs not only when stored in air (in the presence of water vapour), but also when extracted from columns after obtaining the chromatographic data described below (the work involved end-capping, packing of columns, their elution with phosphate buffer solutions of pH 3–7 and polar organic solvents over a period of 2 months). As a result, no decrease in the intensities of these bands was observed.

These unexpected findings led to more detailed IR spectrometric studies of the behaviour of ester surface compounds both on Silasorb 600 microporous silica gel and on non-porous pyrogenic silica (specific surface area 300 m^2/g). In particular, it was ascertained that, in addition to amide groups, ester chemisorption products also form on the surface of non-porous AP-silica (Fig. 6) as a result of the gas-phase acylation

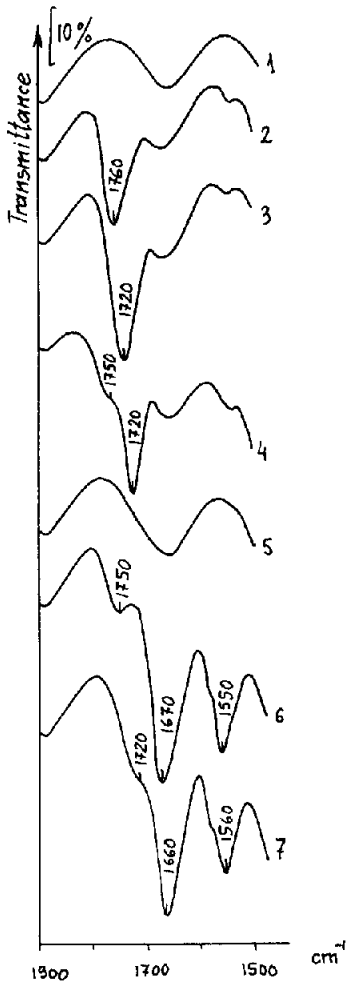

Fig. 6. Infrared spectra of (1) non-porous silica untreated, (2) gas-phase acylated with benzoyl chloride at 170°C and (3) air-exposed for 20 min; (4) microporous silica gel gas-phase acylated with benzoyl chloride at 170°C and air-exposed; (5) non-porous AP-silica untreated, (6) gas-phase acylated with benzoyl chloride at 170°C and (7) air-exposed for 20 min.

with carboxylic acids and benzoyl chloride. However, they turned out to be stable only under vacuum (Fig. 6, curve 6), and in air they hydrolysed in a few minutes (curve 7) to form hydrogen-bonded associations of carboxyl and silanol groups, of which the absorption $\nu_{C=O}$ at 1720 cm^{-1} is characteristic [17]. Ester surface compounds produced by the gas-phase acylation of dehydrated (unmodified) silica are also hydrolytically unstable (curves 2 and 3). At the same time only part of the ester products are hydrolysed ($\nu_{C=O}$ at 1720 cm^{-1}) on microporous silica gel (curve 4), both on exposure to a humid atmosphere and on immersion into water, while the $\equiv$SiOCO– band remains as a shoulder at 1750 cm^{-1}. It follows that only those ester products

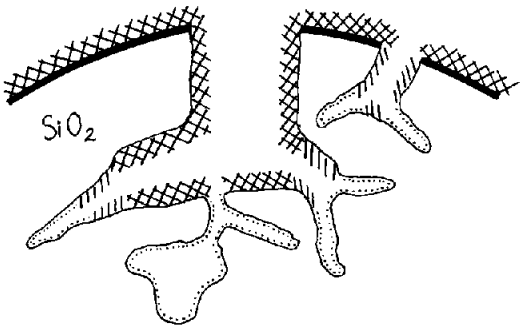

Fig. 7. Schematic diagram of acylamide SP surface fragment: dotted region, micropores inaccessible to reagent molecules; cross-hatched region, silanized surface; and single-hatched region, zones containing hydrolytically stable ester products.

which form in narrow pores of silica gel are hydrolytically stable. Hydrocarbon radicals appear to hydrophobize and "block" these pores, making them inaccessible to water.

On the basis of the above, the structure of the modifying coating of the SPs obtained can be represented schematically as follows. Three types of zones exist on the silica gel surface: (1) surface of micropores inaccessible both to APTES molecules and to the acylating reagents used; (2) surface of narrow pores inaccessible to bulky silane molecules, but permeable to compact molecules of benzoic acid and its chloride and to linear molecules of stearic acid; after formation of ester surface compounds these zones become inaccessible also to water; and (3) surface of broad pores and outside surface of silica gel particles, whereon the silanizing coating forms and its further modification is effected. It is the third type of zone that appears to be responsible for the chromatographic properties of the SPs obtained.

The presence of acylation by-products on the surface of the synthesized SPs prevents an accurate evaluation of the extent of transformation of amino groups into amide groups. Based on the elemental analysis data (Table I), it can only be stated that the resulting concentration of bonded acyl groups with benzoyl chloride used for derivatization is approximately twice that obtained with benzoic and stearic acids.

TABLE I

ELEMENTAL ANALYSIS DATA AND CONCENTRATION OF BONDED GROUPS FOR GAS-PHASE MODIFIED STATIONARY PHASES

SP	Acylating reagent	C(%)	N(%)	H(%)	Concentration of bonded groups, based on C(%)	
					mmol/g	μmol/m^2
APS	–	8.25	1.51	0.84	1.15	2.09
BAPS-C	Benzoyl chloride	18.10	0.95	1.58	1.17	2.13
BAPS-A	Benzoic acid	13.36	1.13	1.21	0.61	1.11
SAPS	Stearic acid	22.52	0.71	3.64	0.66	1.20

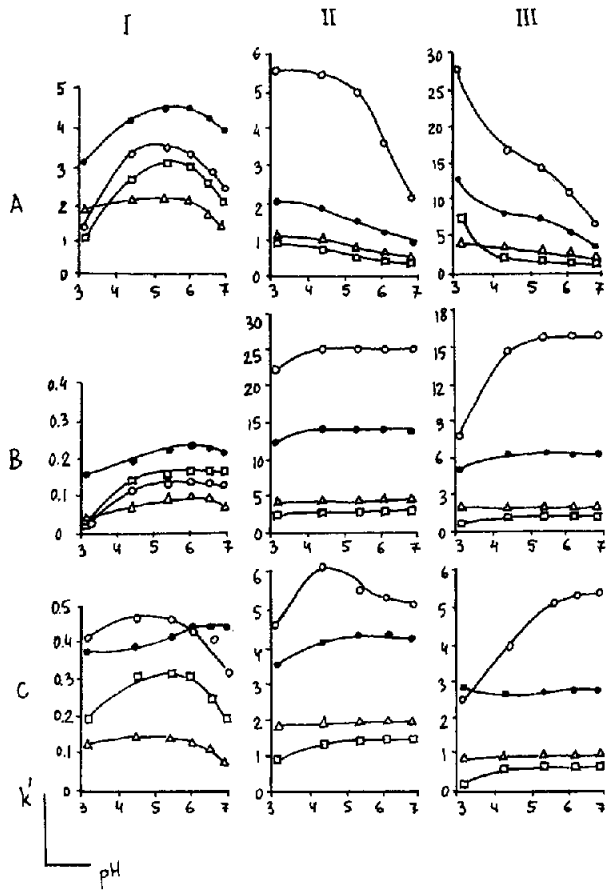

Fig. 8. Plot of retention (k') of nucleic acid constituents by (I) APS, (II) Separon C_{18} and (III) SAPS *vs.* eluent pH. (A) Nucleoside 5'-monophosphates: ○, AMP; ●, GMP; △, UMP; □, CMP. (B) Nucleosides: ○, adenosine; ●, guanosine; △, uridine; □, cytidine. (C) Nucleobases: ○, adenine; ●, guanine; △, uracil; □, cytosine. Column, 64 × 2 mm I.D.; eluent, phosphate buffer at 100 μl/min; temperature, ambient; detection, UV at 270 nm.

Since in the derivatization of aminoorganosilicas with acyl halides the yield of surface amides is close to 100% (see, *e.g.*, ref. 18), for the carboxylic acids in question it can be assumed to amount to about 50%. According to our results (unpublished), such yields of surface acylation products are observed also with aliphatic dicarboxylic, phthalic, aminobenzoic and α-amino acids.

Retention of nucleic acid components

Bonded SPs, containing amine and amide modifying groups, turned out to be fairly convenient for the fractionation of nucleic acids [4,19]. The separation of these compounds is improved owing to a concurrent action of the ionic and the hydrophobic mechanisms of retention. To evaluate the ion-exchange and reversed-phase (RP) properties of the SPs obtained, we studied the retention on them of nucleosides

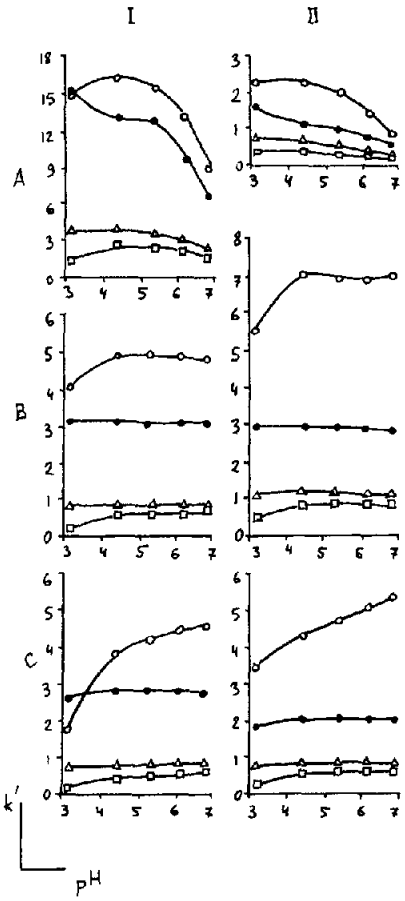

Fig. 9. Plot of retention (k') of nucleic acid constituents on (I) BAPS-A and (II) BAPS-C *vs.* eluent pH. Symbols and conditions as in Fig. 8.

5′-monophosphates, AMP, GMP, CMP, UMP and of the corresponding nucleosides and free bases at various pH values of the eluent.

The k' *versus* pH curves of these compounds for SAPS and, for comparison, APS and a traditional ODS sorbent (Separon C_{18}) are presented in Fig. 8. The elution order of bases for SAPS and Separon C_{18} (cytosine, uracil, guanine, adenine, nucleosides and monophosphates) is the same. For APS in the major part of the studied pH range it is uracil, cytosine, guanine, adenine, U, A, C, G, and UMP, CMP, AMP, GMP, respectively. For free bases the maximum k' values (1–6) were obtained with Separon C_{18} and lower values with SAPS. With APS the k' values were less than 0.5 for all bases over the entire pH range of 3–7. The retention of nucleosides on SAPS and Separon C_{18} (maximally) was also approximately one order of magnitude higher than for APS. Finally, the highest k' values for most of monophosphates, differing greatly from those on Separon C_{18} and APS, were obtained on SAPS. Thus, whereas SAPS behaves as a typical ODS sorbent in the separation of electroneutral compounds (nucleosides and

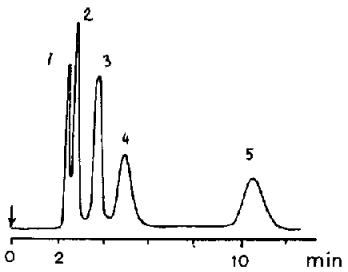

Fig. 10. Separation of nucleosides on BAPS-C: 1 = cytidine; 2 = uridine; 3 = inosine; 4 = guanosine; 5 = adenosine. pH, 4.40; other conditions as in Fig. 8.

corresponding bases), both hydrophobic and ionic interactions with the SP surface contribute to the retention of nucleotides.

BAPS-A also exhibits similar chromatographic properties with respect to nucleic acid components (Fig. 9). There are a number of insignificant differences from SAPS (shape of curves, specific k' values). Deserving more attention, in our opinion, is the fact that this SP, in contrast to BAPS-C, which is close to it in the structure of the bonded layer, exhibits multiphase properties. The ion-exchange properties of BAPS-C turned out to be suppressed because of the use of a very active acylating reagent, benzoyl chloride, in the gas-phase modification and a virtually complete transformation of amino groups into benzamide derivatives. This is evidenced by the fact that the retention of nucleoside 5′-monophosphates on this SP, in contrast to BAPS-A and SAPS, has very low values (Fig. 9) and, moreover, the elution order of the components under analysis remains unchanged over the entire pH range of 3–7. For the rest, BAPS-C is similar to BAPS-A, but exhibits a better selectivity in the separation of nucleosides and free bases. An example of the separation of a mixture of nucleosides is presented in Fig. 10.

Separation of amino acid enantiomers

The following approach is very convenient for the separation of amino acid racemates on ODS sorbents. The C_{18} SP is treated *in situ* with a solution of a derivative of an optically active amino acid, containing a long (C_7–C_{18}) linear hydrocarbon radical [15,16]. Such compounds are strongly sorbed and not washed away by aqueous eluents. Enantiomers of many amino acids can be separated with high selectivity coefficients on the SP thus obtained by ligand-exchange chromatography.

We ascertained that a considerable concentration (about 50% of the initial value) of polar AP groups on the SAPS surface does not affect the capability of bonded stearic acid residues for firm retention of an optically active modifier of such a type, N-octyl-L-proline. This proline derivative is strongly sorbed both on a traditional ODS sorbent (Separon C_{18}) and on SAPS, building in the octyl chain between hydrophobic radicals of the SP (Fig. 11).

After saturation of dynamically modified SPs with copper(II) ions [15,16], we carried out the separation on them of racemates of a number of hydrophobic amino acids. All the steps of modification of both SPs, their saturation with copper(II) ions and the separation of racemates were conducted under identical conditions, in the same sequence and with use of the same reagents.

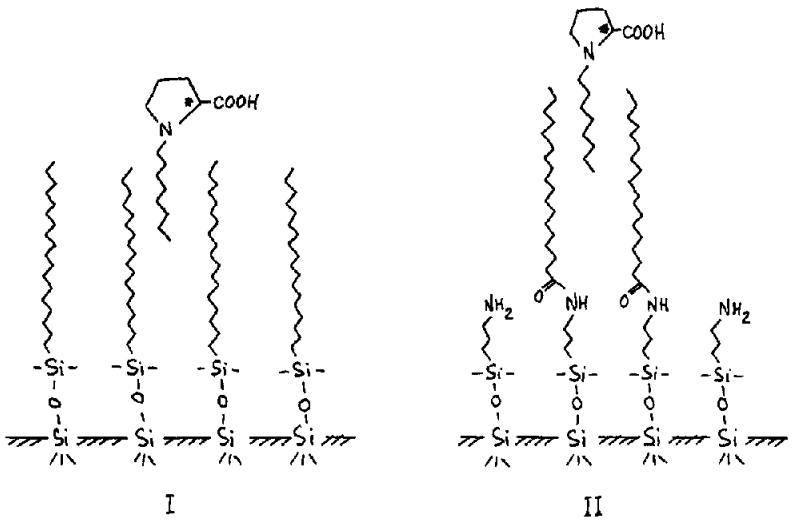

Fig. 11. Coating of (I) Separon C_{18} and (II) SAPS with N-octyl-L-proline.

L-Enantiomers were eluted first in all instances. This is accounted for by the fact that only D-enantiomers are capable of incorporating their side radicals between alkyl groups of reversed phases, whereas α-substituents of L-enantiomers are only attracted to hydrophobic substrate, owing to which the stability of diastereomeric L L complexes turns out to be lower than that of D–L complexes [15,20].

Retention parameters and selectivity coefficients for some enantiomer pairs are presented in Table II, from which it is seen that a high selectivity of separation is attained on both SPs with the use of the simplest eluent, water with an addition of a copper salt. The minimum $\alpha_{D/L}$ values were obtained for methionine, *viz.*, 1.62 (C_{18}) and 1.68 (SAPS), eluent 10^{-3} *M* $CuSO_4$, and the maximum values for leucine, *viz.*, 2.51 (C_{18}) and 3.24 (SAPS), eluent 10^{-4} *M* $CuSO_4$. Higher k' and α values for most enantiomer pairs were obtained on SAPS than on C_{18}. We believe that the k' increase stems from the fact that, apart from complexation with coordination-unsaturated copper ions, hydrophobic interactions with residual AP groups of SAPS (as in the case of nucleotides) contribute to the retention of amino acids. The $\alpha_{D/L}$ increase is probably also associated with a large number of AP groups on this SP, which restrict the reversed-phase capability for retaining molecules of the dynamic modifier. Thus, the N-octyl-L-proline concentration on the SAPS surface turns out to be lower than that on Separon C_{18}; this can appropriately be compared with the "dilution" of chemically bonded chiral groups [21,22], which increases the enantioselectivity. Examples of the separation of enantiomers of some hydrophobic amino acids on SAPS dynamically modified with N-octyl-L-proline are presented in Fig. 12.

Separation of oligomers

As a further example of the application of the acylamide SPs produced by the gas-phase modification, we present the separation of oligomers of N-(2,3-epoxypropyl)carbazole (EPC), used in xerography. Analysis of the oligomeric composition

TABLE II

COMPARISON OF k' AND α VALUES OF SOME AMINO ACID ENANTIOMERS ON N-OCTYL-L-PROLINE-COATED SEPARON C_{18} AND SAPS

Conditions as in Fig. 12. Mobile phases: (I) 10^{-4} and (II) 10^{-3} M $CuSO_4$.

Amino acid	Mobile phase	Separon C_{18}			SAPS		
		k'_L	k'_D	$\alpha_{D/L}$	k'_L	k'_D	$\alpha_{D/L}$
Norleucine	II	3.63	7.00	1.93	5.00	11.00	2.20
Leucine	I	2.32	5.82	2.51	4.83	15.67	3.24
	II	2.27	5.36	2.36	3.58	8.41	2.35
Norvaline	II	1.13	2.36	2.09	1.46	3.92	2.68
Valine	I	1.50	3.64	2.42	1.50	4.17	2.78
	II	1.05	2.18	2.08	1.50	3.58	2.39
Tyrosine	I	3.40	7.33	2.16	10.17	21.08	2.07
	II	3.05	5.91	1.94	5.50	12.91	2.35
Phenylalanine	II	11.18	18.27	1.63	4.41	12.42	3.22
Methionine	I	3.95	7.45	1.89	3.17	5.33	1.68
	II	1.91	3.09	1.62	2.83	4.75	1.68

of EPC on the ODS SP demonstrates the presence of chains containing 2–13 monomeric units (Fig. 13B). No separation occurs on BAPS-A; all components are retained very weakly. A partial separation is achieved on BAPS-C (Fig. 13C). The application of SAPS, despite the presence of about the same number of residual AP

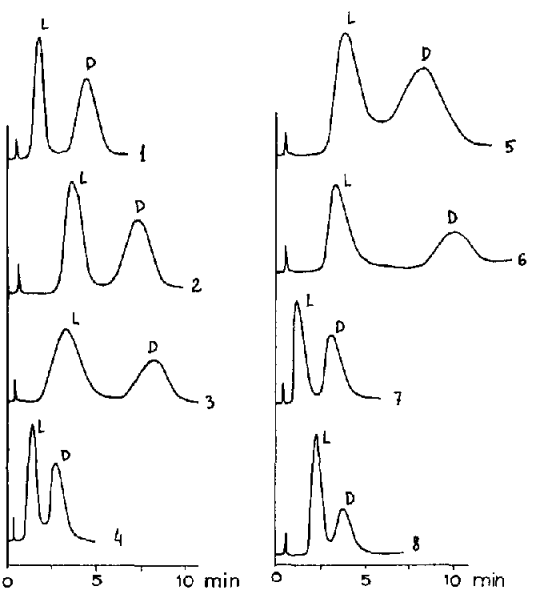

Fig. 12. Enantiomeric resolution of hydrophobic amino acids on N-octyl-L-proline-coated SAPS. 1 = Norvaline; 2 = norleucine; 3 = phenylalanine; 4 and 7 = valine; 5 = tyrosine; 6 = leucine; 8 = methionine. Column, 64 × 2 mm I.D.; eluents, (1–5) 10^{-3} and (6–8) 10^{-4} M $CuSO_4$ at 220 μl/min; temperature, ambient; detection, UV at 240 nm.

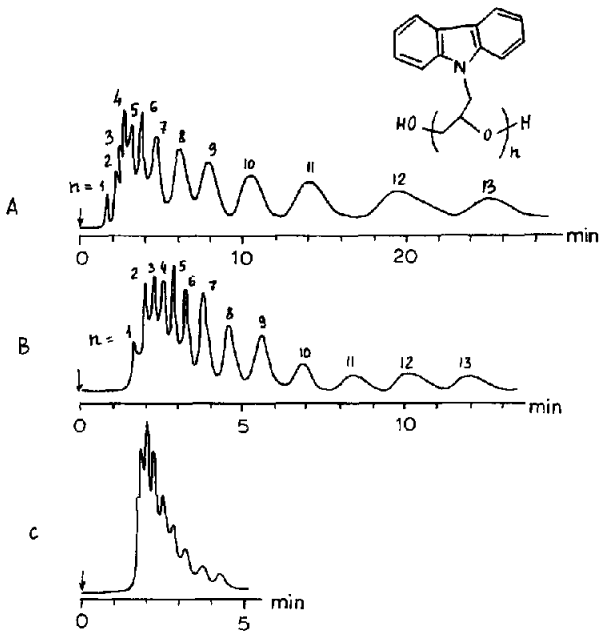

Fig. 13. Separation of EPC oligomers on (A) SAPS, (B) Separon C_{18} and (C) BAPS-C; 1 = N-(2,3-epoxypropyl)carbazole (monomeric); 2 = dimer; etc. Column, 64 × 2 mm I.D.; eluent, acetonitrile at 100 µl/min; temperature, ambient; detection, UV at 300 nm.

TABLE III

COMPARISON OF k' AND α VALUES OF EPC OLIGOMERS ON SEPARON C_{18} AND SAPS

Conditions as in Fig. 13.

n	Separon C_{18}		SAPS	
	k'_n	$\alpha_{n+1/n}$	k'_n	$\alpha_{n+1/n}$
1 (monomeric)	0.42		0.15	
		1.74		4.33
2	0.73		0.65	
		1.17		1.42
3	0.86		0.92	
		1.30		1.29
4	1.12		1.19	
		1.24		1.16
5	1.39		1.38	
		1.26		1.42
6	1.75		1.96	
		1.28		1.37
7	2.24		2.67	
		1.27		1.40
8	2.85		3.77	
		1.29		1.35
9	3.67		5.08	
		1.26		1.45
10	4.63		7.38	
		1.28		1.32
11	5.92		9.77	
		1.24		1.46
12	7.33		14.23	
		1.23		1.34
13	9.00		19.00	
$\bar{\alpha}_{n+1/n}$		1.26		1.36

groups as on the surface of BAPS-A, yielded good results (Fig. 13A); all the compounds under analysis are well retained and are separated with high selectivity coefficients. The $\alpha_{n+1/n}$ values obtained in the separation of EPC on SAPS exceed the corresponding values for Separon C_{18} by a factor of 1.08 on average (Table III).

Hence the gas-phase modification of amino phases with carboxylic acids is an unlaborious, rapid and efficient method for the synthesis of acylamide SPs, which requires no activation of carboxylic groups or the use of condensing reagents. As the yield of amino groups acylation is about 50%, these phases contain both hydrophobic and anion-exchanging groups, which can contribute to the separation process simultaneously; hence such SPs are convenient for the separation of various classes of compounds.

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