MULTIPARAMETRIC CHARACTERIZATION OF AMINO ACIDS- AND PEPTIDE-SILICA STATIONARY PHASES – A COLUMN SELECTION FOR SEPARATION TARGETS

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INTRODUCTION

In terms of the separation techniques that are widely used in many fields of science, the choice of stationary phase which are suitable for our separation targets represents an imperative objective. Thus, the characterization of surface properties that result from a specificity of chemically bonded ligands and their impact on the overall chromatographic behavior is essential.

The immobilization of suitable amino acids sequences on the silica surface allows obtaining stationary phases with different hydrophobicity and polarity. The appropriate selection of amino acids and peptide sequences allows the preparation of stationary phases useful in desired chromatographic systems. In order to prove this assumption and facilitate the column selection for the potential application, the description of the structure-selectivity relationships for newly synthesized stationary phases must be performed.

The aim of the research was to carry out a multiparametric characterization of nine home-made stationary phases with chemically bonded amino acids and peptides. These materials were characterized in terms of the selectivity for hydrophilic and hydrophobic compounds. The applied column characterization methodology allowed the classification of the tested stationary phases according to their chromatographic properties. Based on this specification, amino acids and peptide-silica stationary phases were successfully applied in analysis of biologically significant compounds.

EXPERIMENTAL

Chromatographic experiments were performed on the Shimadzu Prominence* and Shimadzu UHPLC Nexera** LC systems (Kyoto, Japan) equipped with ternary* and binary** gradient pump, an autosampler, a diode array detector, and column thermostat. Instrument control, data acquisition, and processing were performed with LabSolutions software for HPLC. The methodology was based on the investigation of differences in selectivities of the tested materials for certain pairs of compounds, which provide specific interaction modes. Working solutions comprised selected pairs of compounds as well as toluene (HILIC) and thiourea (RP HPLC) as a void volume markers (Table 1).

STRUCTURES

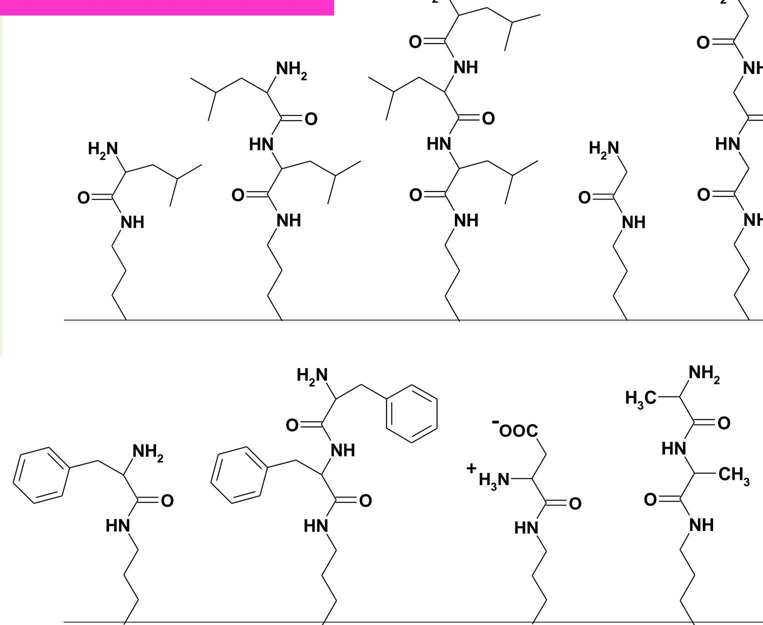

Fig. 1. Structures of chemically bonded stationary phases: A – amino-$(Leu)_1$, B - amino-$(Leu)_2$, C -amino-$(Leu)_3$, D - amino-$(Gly)_1$, E - amino-$(Gly)_3$, F - amino-$(Phe)_1$, G - amino-$(Phe)_2$, H - amino-$(Asp)_1$, I – amino-$(Ala)_2$.

Table 1 Operation parameters of chromatographic tests and properties of tested compounds

Mix no.	Tested solutes	Sign	Molecular stucture	pKa	LogD LogP *	Variable	Mobile phase composition
HILIC mode							
1	Uridine – 5-methyluridine	α (CH_2)		12.6 12.0	-1.58 -1.02	Hydrophobic interactions	ACN/20 mM NH_4Ac pH = 4.7 $(90/10_{v/v})$
2	Uridine – 2`-deoxyuridine	α (OH)		12.6 13.9	-1.58 -1.26	Hydrophilic interactions	
3	Vidarabine – adenosine	α (V/A)		13.9 13.9	-1.02 -1.03	Configuratio-nal isomers selectivity	
4	2`-deoxyguanosine – 3`-deoxyguanosine	α (2dG/3dG)		13.5 13.5	-1.14 -1.14	Regioisomers selectivity	
5	Sodium p-toluenesulfonate – uracil	α (AX)		-12.8 13.8	0.88 -1.08	Anion exchange selectivity	
6	N,N,N-trimethylphenylammonium chloride - uracil	α (CX)		- 13.8	-2.31 -1.08	Cation exchange selectivity	
7	Theobromine – theophylline	α (Tb/Tp)		10 8.6	-1.06 -2.51	Acidic-basic nature of stationary phase	
RP mode							
1	Pentyl- and butylbenzene	α (PB/BB)		-	5.0 4.4	Hydrophobi-city	ACN/H_2O $(30/70_{v/v})$
2	Trifenylen – *o*-terfenyl	α (T/O)		-	4.9 5.5	Steric selectivity	
3	Caffeine – phenol	α (C/P)		10.4 9.99	-0.1 1.46	Hydrogen bonding capacity	$MeOH/H_2O$ $(30/70_{v/v})$
4	Benzylamine – phenol	α $(B/P)_{7.6}$		9.34 9.99	1.1 1.46	Ion-exchange capacity at pH > 7	MeOH/20 mM KH_2PO_4 pH = 7.6
5		α $(B/P)_{2.7}$				Ion-exchange capacity at pH < 3	MeOH/20 mM KH_2PO_4 pH = 2.7

* Value presented in this column correspond to logD of particular solutes in the case of HILIC test, while in RP conditions logP values were provided.

RESULTS

HILIC

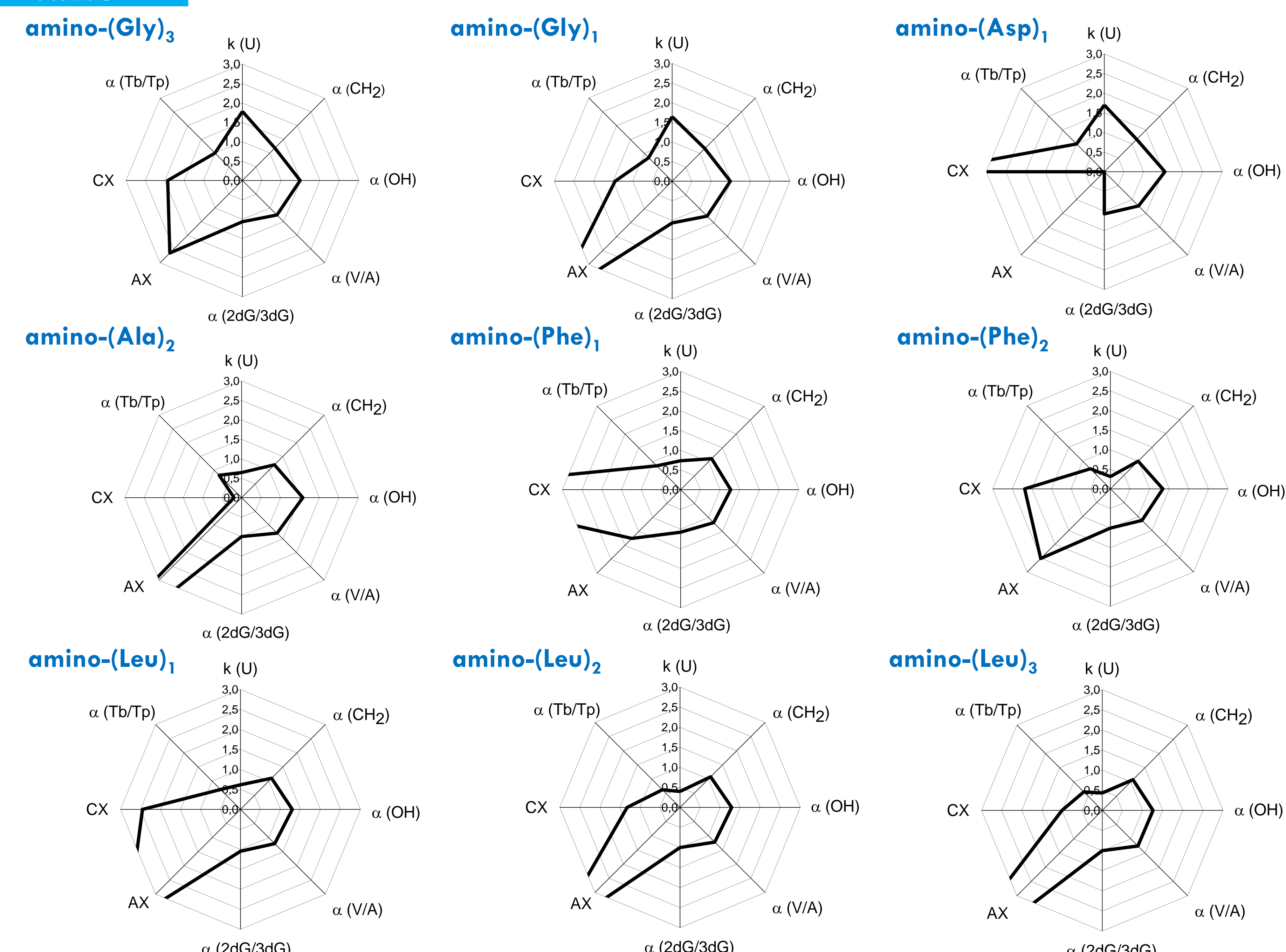

Fig. 2. Radar plots of characterization data – the comparison of investigated stationary phases.

RP HPLC

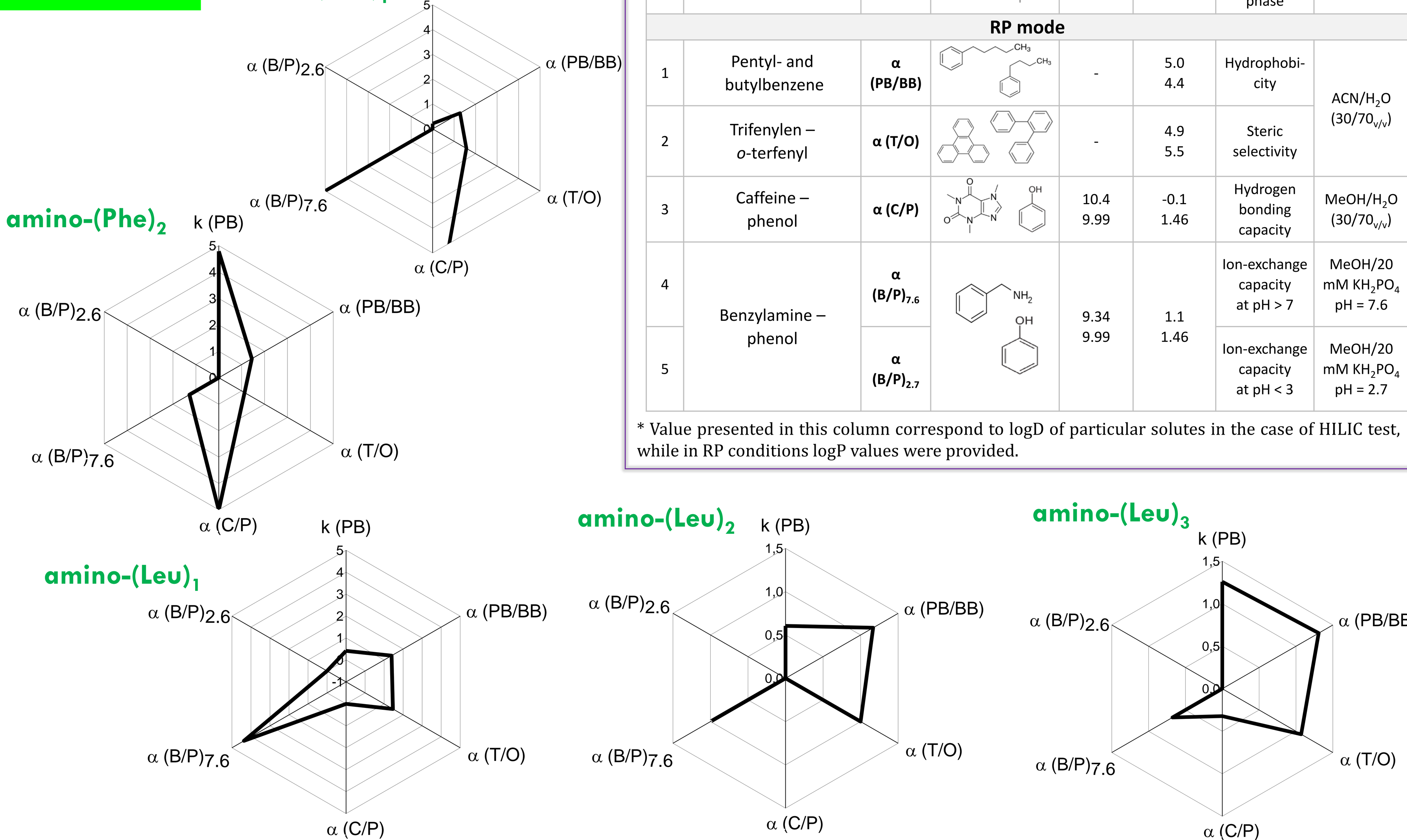

Fig. 3. Radar plots of hydrophobic stationary phases – the comparison of RP-characteristic properties.

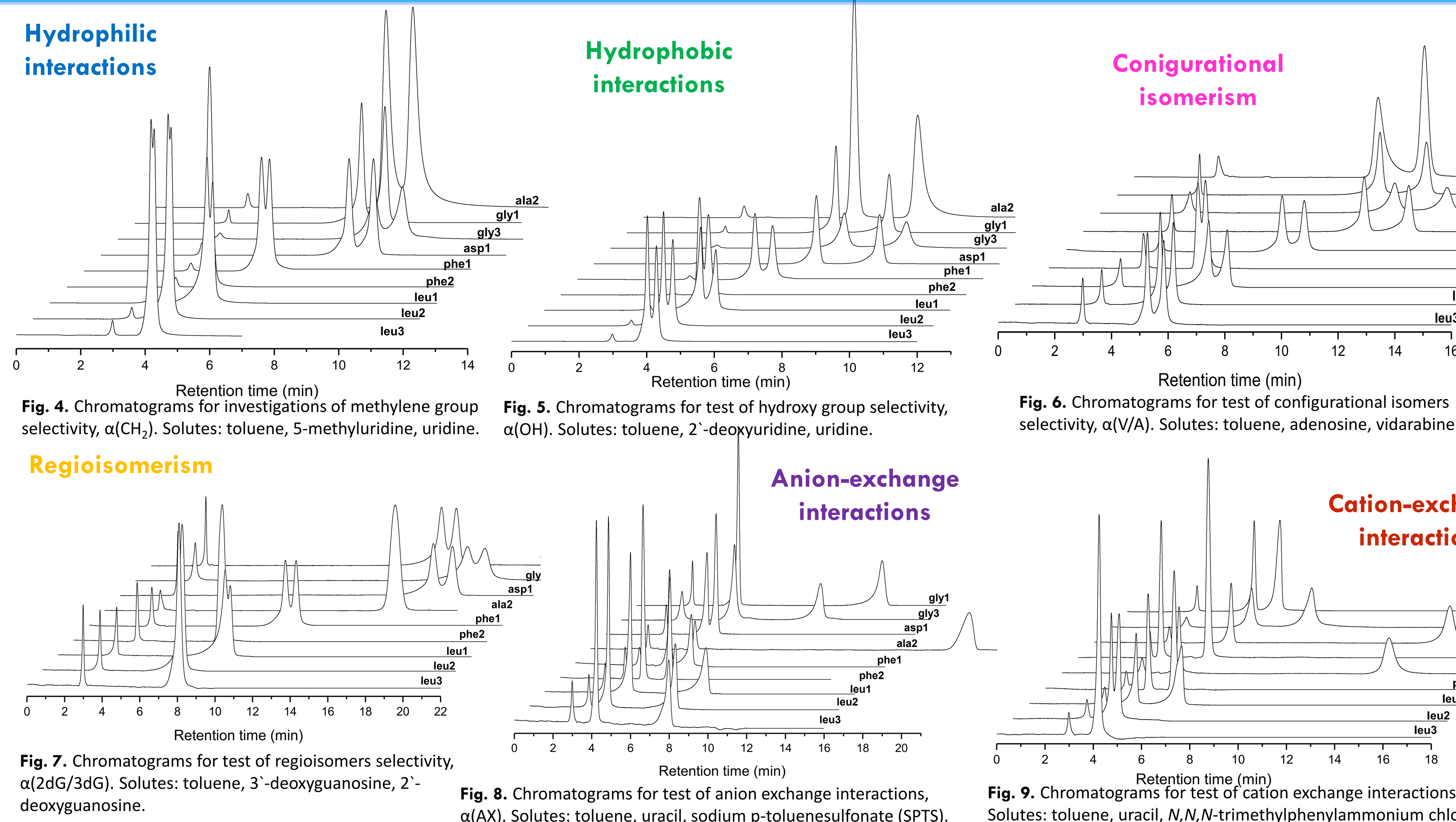

Fig. 4. Chromatograms for investigations of methylene group selectivity, α(CH_2). Solutes: toluene, 5-methyluridine, uridine.

Fig. 5. Chromatograms for test of hydroxy group selectivity, α(OH). Solutes: toluene, 2`-deoxyuridine, uridine.

Fig. 6. Chromatograms for test of configurational isomers selectivity, α(V/A). Solutes: toluene, adenosine, vidarabine.

Fig. 7. Chromatograms for test of regioisomers selectivity, α(2dG/3dG). Solutes: toluene, 3`-deoxyguanosine, 2`-deoxyguanosine.

Fig. 8. Chromatograms for test of anion exchange interactions, α(AX). Solutes: toluene, uracil, sodium p-toluenesulfonate (SPTS).

Fig. 9. Chromatograms for test of cation exchange interactions, α(CX). Solutes: toluene, uracil, *N,N,N*-trimethylphenylammonium chloride.

APPLICATIONS

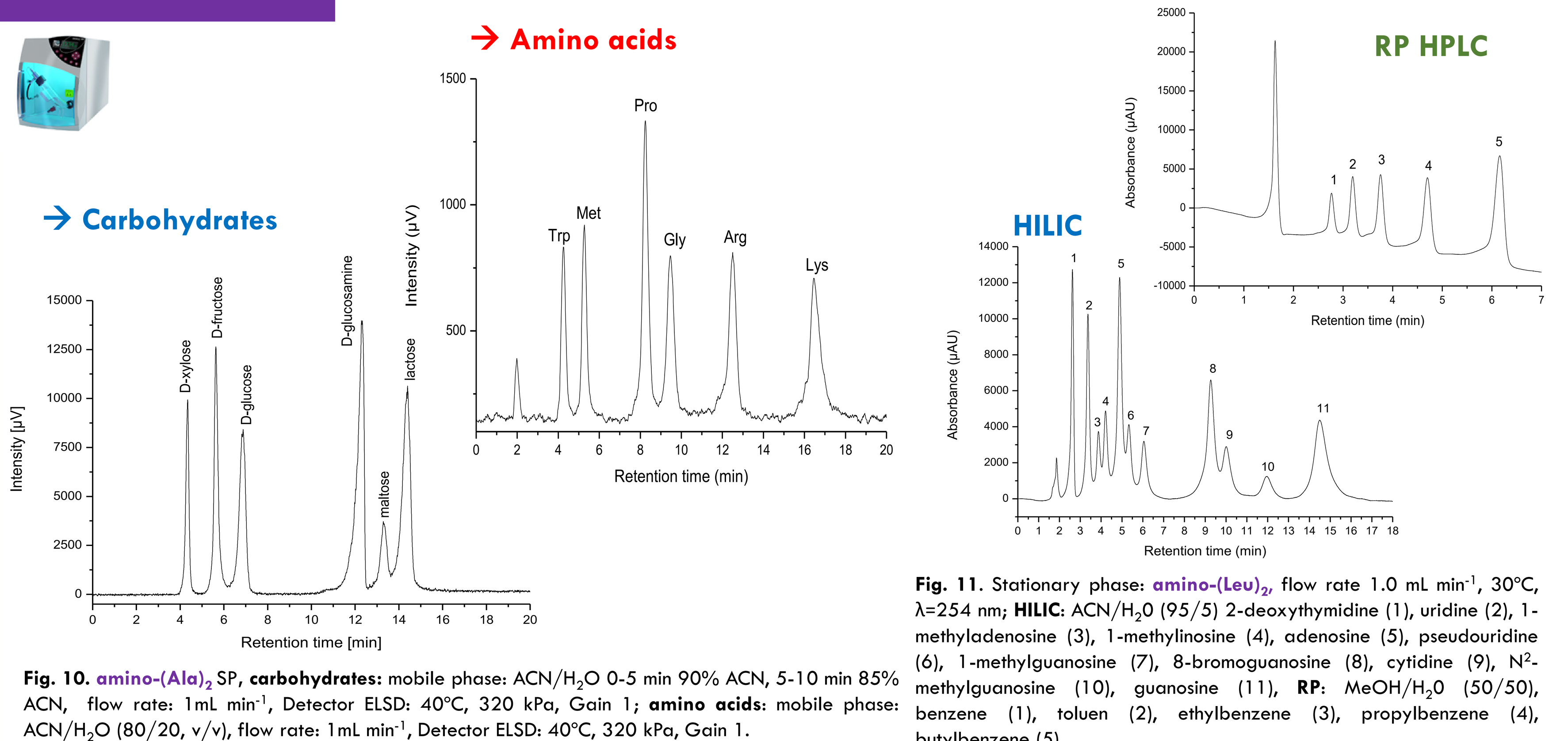

Fig. 10. amino-$(Ala)_2$ SP, **carbohydrates:** mobile phase: ACN/H_2O 0-5 min 90% ACN, 5-10 min 85% ACN, flow rate: 1mL min^{-1}, Detector ELSD: 40°C, 320 kPa, Gain 1; **amino acids**: mobile phase: ACN/H_2O (80/20, v/v), flow rate: 1mL min^{-1}, Detector ELSD: 40°C, 320 kPa, Gain 1.

Fig. 11. Stationary phase: amino-$(Leu)_2$, flow rate 1.0 mL min^{-1}, 30°C, λ=254 nm; **HILIC**: ACN/H_2O (95/5) 2-deoxythymidine (1), uridine (2), 1-methyladenosine (3), 1-methylinosine (4), adenosine (5), pseudouridine (6), 1-methylguanosine (7), 8-bromoguanosine (8), cytidine (9), N^2-methylguanosine (10), guanosine (11), **RP**: $MeOH/H_2O$ (50/50), benzene (1), toluen (2), ethylbenzene (3), propylbenzene (4), butylbenzene (5).

CONCLUSIONS

In terms of the absolute hydrophilicity, tested materials may be divided into two groups. Stationary phases containing glycine, alanine, and aspartic acid in amino acids sequence demonstrated higher hydrophilic retention than modifications with leucine and phenylalanine. These correlations were in compliance with the hydrophilic/hydrophobic nature of bonded amino acids. It should emphasized that despite of the lower polarity of the second group of materials, they are compatible for HILIC applications. The discrimination of configurational isomers was comparable for all the investigated stationary phases, while the regioisomers was subtly distinguished by materials with immobilized hydrophilic amino acids and peptides. The anion-exchange capability was observed for all the tested columns except the stationary phase with aspartic acid. The presence of carboxyl group in the side chain of such amino acid plays as cation-exchange functionality, simultaneously causes the electrostatic repulsion with anionic compound.

Stationary phases with chemically bonded hydrophobic amino acids and peptides (leucine and phenylalanine) demonstrated also the RP-compatible character. Among the stationary phases investigated, material with bonded dipeptide of phenylalanine exhibits the greatest hydrophobicity. Moreover, the retention of hydrophobic solutes increased with the elongation of peptide chain. The steric selectivity was slightly higher for single amino acid modification compared to peptide ligands. In addition, the ion-exchange capacity (caused by residual silanols) was reduced, whereas the peptide chain of particular amino acid was elongated. Judging from these research, stationary phases with immobilized hydrophobic sequence of amino acids could be applied both in RP and HILIC systems.

As a result of the research, it was evident to realize how the sequence of amino acids - their type and length influences on the overall chromatographic properties. This format may comprise convenient approach for column selection depending on HILIC or RP HPLC separation targets.

Acknowledgments

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