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High-performance liquid chromatography of amino acids and dipeptides on new ion exchangers of the HEMA series

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ABSTRACT

The separation of a number of essential amino acids and dipeptides was studied in ion-exchange systems based on poly(hydroxyethyl methacrylate) stationary phases modified with various ionizing groups (carboxymethyl, a weak cation exchanger; sulphobutyl, a strong cation exchanger; diethylaminoethyl, a weak anion exchanger) and on an ethylene dimethacrylate-based hydrophobic column. The ion-exchange materials also exhibited hydrophobic properties, caused by the poly(hydroxyethyl methacrylate) matrix. The dependence of the capacity factors on the mobile phase pH, the ionic strength, the type and concentration of the displacer salt and the content of the organic modifier (methanol) was investigated. The best separations of amino acids and dipeptides, with baseline resolution for many test compounds, was attained with cation exchangers, HEMA Bio 1000 CM and SB. The analytes were detected either by measurement of the absorbance at 210 nm or amperometrically at a copper electrode, at a potential of +0.15 V (Ag/AgCl) (for separations at pH > 6).

INTRODUCTION

High-performance liquid chromatography (HPLC) has proved to be a highly efficient method for separation of amino acids and peptides [1,2]. A great variety of separation modes have been investigated, primarily reversed-phase, ion-exchange and size-exclusion systems, reversed-phase chromatography being the most common. It has been shown [3] that C_8 columns are more efficient than C_{18} columns for the separation of peptides. Ion-exchange columns have the advantages of large loading capacities and high resolving power [4,5], but long-chain peptides are difficult to separate because they are strongly bound to the stationary phase matrix [6]. Size-exclusion separations are applicable to peptides in a wide molecular-weight range of *ca.* $100–5 \cdot 10^6$ dalton [7,8]; the analytes are often resolved better than in reversed-phase systems, but their behaviour is far from the theoretical assumptions [8], which complicates the prediction and optimization of the experimental conditions.

The retention of amino acids and especially peptides is primarily affected by the mobile phase pH because of the well known, complex acid–base properties of these

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compounds and because of the obvious effect of the pH in the ion-exchange systems. The ionic strength of the mobile phase and the content of an organic modifier exert a smaller effect through variation of the mobile phase polarity and solubility of the analytes. The type of displacer salt and its concentration affect the ion-exchange separations.

The reversed-phase mechanism is most complex. The retention of peptides usually increases with increasing pH (*e.g.* with peptide-bonded phases [9]): at low pH values, amino acids and peptides have a net positive charge owing to protonation of the amino groups, which suppresses the analyte solubility in the mobile phase but also decreases their affinity for the non-polar stationary phase. The presence of an organic modifier usually causes a decrease in the retention time [10], owing to improved solubility of the analytes in the mobile phase, but log k' (capacity factor) *versus* modifier content dependences exhibiting a minimum have also been reported [11].

Ion-pairing agents have been used extensively in reversed-phase separations and, for example, the retention of positively charged amino acids and peptides increased with increasing concentration of trifluoroacetate or phosphate used as ion-pairing agents [12–14]. It is also possible to predict the retention times for a particular ion-pairing agent [12]. The effect of the counter-ion depends on its hydrophobicity [12].

A number of authors [15–21] have derived empirical rules for prediction of the retention of peptides in reversed-phase systems. Retention coefficients have been defined to describe the effect of the individual amino acid residues [15]: it has been found that an individual set of the retention coefficients is necessary for each position of an amino acid within the peptide [22]. Reversed-phase separations are usually more efficient and faster than ion-exchange separations, except for hydrophilic amino acids and peptides that are not retained [23]. Non-ionic polyethylene alcohol surfactants can be used to control the retention of polypeptides in reversed-phase systems through variation of the mobile phase surface tension [24].

Amino acids and peptides are difficult to detect. Either the analytes are derivatized, or very low wavelengths must be used (200–220 nm) for UV photometry, except for the substances containing aromatic systems, which absorb around 280 nm. The terminal amino groups and aromatic systems can be electrochemically oxidized [25]. Detection based on chelate formation at a passivated copper electrode [13,14,26–28] is a very convenient, selective and sensitive technique for amino acids and short peptides.

The present paper deals with a critical comparison of separations of amino acids and peptides on a newly produced [28] series of ion exchangers, based on a poly-(hydroxyethyl methacrylate) matrix, modified with various ionizable groups and on an ethylene dimethacrylate hydrophobic column. The ion-exchange materials also exhibit hydrophobic and size-exclusion properties, so that there is a possibility of combination of the advantages of various separation modes, as briefly discussed in our previous paper [29].

EXPERIMENTAL

Apparatus

The liquid chromatograph used consisted of an LC-XPD pump and an LC–UV variable-wavelength detector (Pye-Unicam, Cambridge, U.K.), a Rheodyne 7125

injection valve with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.), an ADLC 2 electrochemical detector with a tubular copper electrode [13], an aqueous saturated Ag/AgCl reference and a stainless-steel counter-electrode and a TZ 4620 chart recorder (Laboratorní Přístroje, Prague, Czechoslovakia), and the following stainless-steel columns: HEMA Bio 1000 CM, 7 μm, 80 × 8 mm I.D.; HEMA Bio 1000 SB, 10 μm, 80 × 8 mm I.D.; HEMA Bio 1000 DEAE, 10 μm, 80 × 8 mm I.D.; EDMA, 10 μm, 80 × 8 mm I.D. (all from Tessek, Prague, Czechoslovakia) and Partisil SCX, 10 μm, 250 × 4.6 mm I.D. (Pye Unicam).

The HEMA Bio 1000 columns are based on a hydrophilized hydroxyethyl methacrylate matrix and have an exclusion limit of 800 000–$2 \cdot 10^6$ dalton. The CM column is a weak cation exchanger with the carboxymethyl ion-exchange group and an exchange capacity of 0.9–1.1 mequiv./g. The SB column is a strong cation exchanger containing the sulphobutyl groups and has an exchange capacity of 1.4–1.8 mequiv./g. The DEAE column is a weakly basic anion exchanger with the diethylaminoethyl group and a capacity of 1.2–1.5 mequiv./g. The EDMA column is hydrophobic, based on an ethylene dimethacrylate matrix. The unmodified HEMA materials exhibit weak hydrophobic interactions.

Chemicals

The standard substances of the amino acids and peptides studied (Gly, Ala, Val, Leu, Ile, Ser, Asp, Glu, Lys, Asn, Cys, Met, Phe, Tyr, His, Trp, Pro, Gly-NH_2, Gly-Gly, Glu-Ala, Gly-Val, Gly-Leu, Gly-Phe, Gly-Tyr, Gly-Asp, Gly-Glu, Gly-His, Gly-Asn and Leu-Gly) were of analytical-grade purity, supplied by Sigma (St. Louis, MO, U.S.A.). The substances were used as received. Stock solutions with a concentration of 0.1 mg/ml were obtained by dissolving the substances in a particular mobile phase and were stored in a refrigerator. The working solutions were prepared immediately prior to measurements by diluting the stock solutions with the mobile phase used.

The mobile phase consisted of an aqueous phosphate buffer (orthophosphoric acid–potassium dihydrogenphosphate) at various concentrations (from 0.001 to 0.020 *M*), at various pH values (from pH 3 to 7, adjusted by additions of orthophosphoric acid and potassium hydroxide solutions), containing varying amount of methanol (between 0 and 20%, v/v) and varying concentrations (from 0.02 to 0.2 *M*) of displacer salts (lithium chloride, sodium chloride, potassium chloride or magnesium chloride). When working with the EDMA column, trifluoroacetic acid was used at various concentrations (from 0.05 to 0.5%) instead of the phosphate buffer.

All the other chemicals used were analytical-reagent grade, obtained from Lachema (Brno, Czechoslovakia) and were not further purified.

Procedure

All the experiments were carried out at a mobile phase flow-rate of 0.5 ml/min and at room temperature (22 ± 2°C). The mobile phases were deaerated by passage of helium. The UV photometric detection was carried out at 210 nm. The amperometric detection at a copper tubular electrode was performed at a potential of +0.15 V (Ag/AgCl, aqueous, saturated), after working electrode activation at −0.3 V (for details see refs. 13 and 27).

The retention data were expressed in terms of the capacity factors, $k' = (t_R - t_M)/t_M$, where t_R and t_M are the retention and dead times, respectively; the dead time was obtained as the solvent peak retention time.

RESULTS AND DISCUSSION

Separation of amino acids

Selected values of the capacity factors on the studied stationary phases are listed in Table I.

The dependence of the capacity factors on the pH (see Fig. 1 for the HEMA Bio 1000 CM column) is similar for the HEMA Bio 1000 CM and SB, *i.e.* the retention decreases with increasing pH. The retention of the amino acids containing negatively charged functional groups (*e.g.* Glu and Asp) is more influenced by the mobile phase pH. The zwitterions formed at higher pH values are generally less retained than the corresponding, positively charged, amino acids at low pH values. It is evident that the amino acid retention on these columns is controlled not only by ion exchange, but also by hydrophobic interactions, as demonstrated in Fig. 1 by the significantly greater retention of Tyr and Phe containing the hydrophobic chains $-CH_2C_6H_4OH$ and $-CH_2C_6H_5$, respectively. For this reason, the retention of the test amino acids on the Partisil SCX column, which is a sole strong cation exchanger, is generally similar and lower than on the HEMA Bio 1000 SB column (see Table I). As expected, the retention of the amino acids on the EDMA column, which exhibits purely hydrophobic interactions, is virtually independent of the mobile phase pH. The retention on the HEMA Bio 1000 DEAE column, a weak anion exchanger, is generally very low, even

TABLE I

CAPACITY FACTORS OF AMINO ACIDS ON THE CATION EXCHANGER STUDIED

Mobile phase: 0.002 *M* potassium dihydrogen phosphate, pH 5.3.

Amino acid	Capacity factor			
	HEMA Bio 1000 CM	HEMA Bio 1000 SB	Partisil SCX	EDMA[a]
Asp	0.11	0.13	0.14	0.25
Asn	0.11	0.14	0.16	0.09
Glu	0.25	0.25	0.16	0.38
Cys	0.51	–	0.84	0.125
Pro	0.53	0.81	1.20	0.25
Ser	0.56	1.19	0.80	0.125
Gly	0.58	1.00	0.44	0.125
Ala	0.58	1.06	0.80	0.156
Val	0.58	1.25	0.84	0.38
Met	0.67	1.88	1.20	1.80
Ile	0.70	1.69	1.10	1.88
Leu	0.70	1.94	0.80	2.06
Phe	1.02	4.12	1.48	7.12
Tyr	1.19	6.00	0.80	6.06
Trp	3.90	27.0	–	>15
His	14.50	>30	–	–

[a] 0.2% trifluoroacetic acid, pH 1.6.

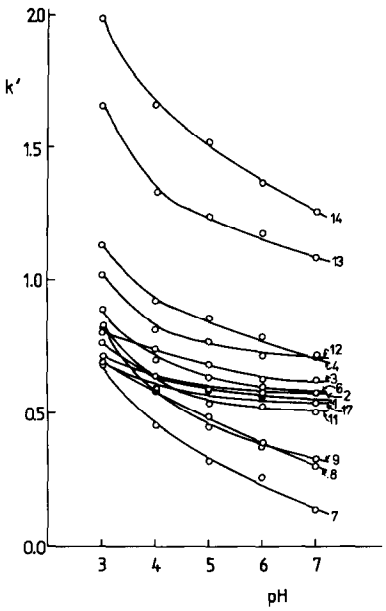
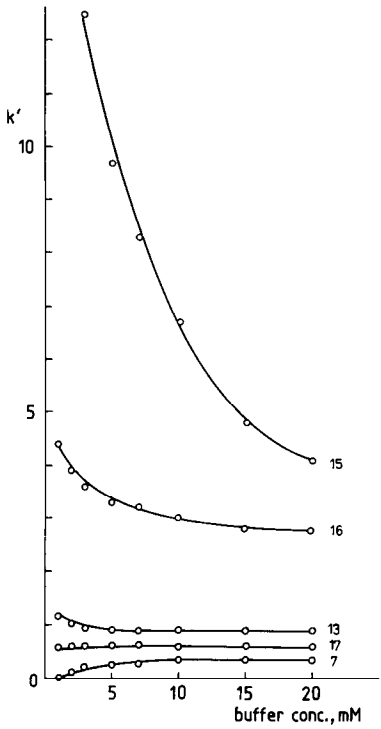


Fig. 1. Dependence of the amino acid capacity factors on the mobile phase pH. HEMA Bio 1000 CM column; mobile phase, $1.5 \cdot 10^{-3}$ *M* aqueous phosphate buffer. 1 = Gly; 2 = Ala; 3 = Val; 4 = Leu; 6 = Ser; 7 = Asp; 8 = Glu; 9 = Lys; 11 = Cys; 12 = Met; 13 = Phe; 14 = Tyr; 17 = Pro.

at high pH values ($\geqslant 9$); this column seems to be unsuitable for separation of compounds of this type and thus was not further studied.

The concentration of phosphate in the mobile phase, *i.e.* the ionic strength, little affects the amino acid retention on the hydrophobic EDMA column (the retention slightly decreases with increasing phosphate concentration, as increasing ionic strength causes a decrease in the mobile phase polarity). The retention on the Partisil SCX column also somewhat decreases with increasing phosphate concentration, as expected for the ion-exchange mechanism. The greatest effect can be seen with the HEMA Bio 1000 CM and SB columns (see Fig. 2 for the CM column). The retention of the positively charged amino acids and those containing hydrophobic groups decreases with increasing phosphate concentration: on the other hand, the retention of negatively charged solute molecules increases with increasing phosphate concentration up to a value of *ca.* $7 \cdot 10^{-3}$ M. This effect again stems from the dual character of the HEMA materials, *i.e.* that of an ion exchanger and a hydrophobic phase. The amino acids are separated into three groups by ion exchange, namely positively charged, negatively charged and uncharged molecules. Within these groups, the elution order is primarily determined by the hydrophobicity and size of the amino acid functional groups, *e.g.* Gly < Ala < Leu < Phe < Tyr ($-H < -CH_3 < -CH_2CH(CH_3) < -CH_2C_6H_5 < -CH_2C_6H_4OH$).

The content of the organic modifier, methanol, in the mobile phase has little effect on the amino acid retention on the ion-exchange columns HEMA Bio 1000 CM, SB and Partisil SCX. The only exceptions are His and Trp: the retention of the former



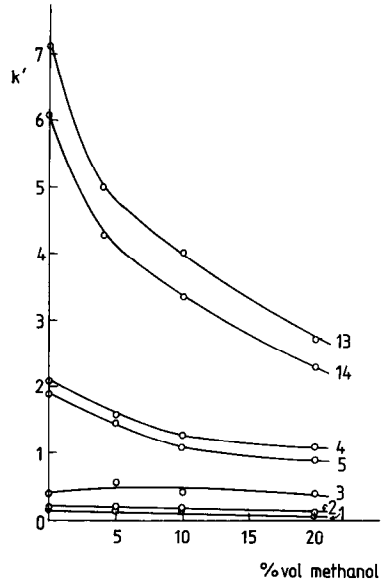


Fig. 2. Dependence of the capacity factors for selected amino acids on the phosphate buffer concentration in the mobile phase. HEMA Bio 1000 CM column; mobile phase, aqueous phosphate buffer, pH 5.3. 7 = Asp; 13 = Phe; 15 = His; 16 = Trp; 17 = Pro; k' values for the other amino acids are generally less than 1.

Fig. 3. Dependence of the capacity factors for selected amino acids on the methanol content in the mobile phase. EDMA column; mobile phase, 0.2% trifluoroacetic acid, pH 1.6. 5 = Ile; other amino acids as in Fig. 1.

increases with increasing methanol content (the k' value on the 1000 CM column changes from 7.1 to 9.1 on an increase in the methanol content from 0 to 15%, v/v), while that of the latter very slightly decreases (the change in k' being from 6.6 to 6.2). On the other hand, the retention on the EDMA hydrophobic column is strongly affected by the methanol content (Fig. 3, Table II). The retention decreases with increasing methanol content, and the effect is more pronounced for more hydrophobic amino acids. It can be seen from Fig. 4 that there exists a linear relationship between log k' and the carbon number in the amino acid functional group, which also confirms the hydrophobic nature of the interactions involved. It is evident that hydrophobic columns represent a promising alternative to ion-exchange and chemically bonded columns in amino acid separations.

Separation of dipeptides

The separation of dipeptides is very similar to that of the amino acids. The

TABLE II

DEPENDENCE OF THE CAPACITY FACTORS OF AMINO ACIDS ON THE METHANOL CONTENT

EDMA column; mobile phase, 0.2% trifluoroacetic acid, pH 1.6.

Amino acid	Capacity factor		
	5% Methanol	10% Methanol	20% Methanol
Asp	0.16	0.19	0.22
Asn	0.16	0.16	0.06
Glu	0.34	0.31	0.28
Cys	0.12	0.12	0.12
Pro	0.25	0.22	0.125
Ser	0.16	0.125	0.09
Gly	0.16	0.125	0.03
Ala	0.28	0.16	0.38
Val	0.66	0.40	0.38
Met	1.44	1.19	0.98
Ile	1.44	1.06	0.88
Leu	1.53	1.25	0.91
Phe	5.00	4.01	2.75
Tyr	4.30	3.38	2.30
Trp	>15	>15	12.4

retention data on the HEMA Bio 1000 CM, SB and Partisil SCX columns are given in Table III. In general, the HEMA Bio 1000 CM and SB columns separate dipeptides better than the Partisil SCX column, for which the capacity ratios are too low. However, with increasing length of the peptides, the capacity factors increase and then

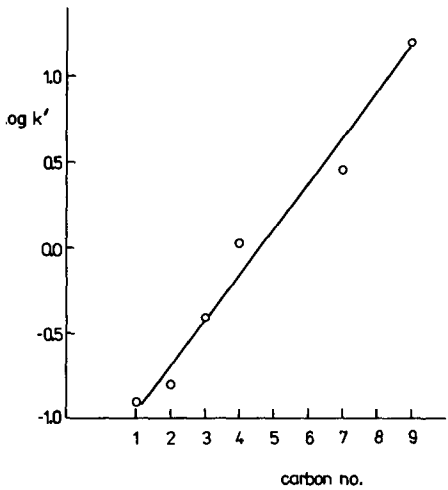


Fig. 4. Dependence of log k' on the carbon number in the amino acid functional groups. Conditions as in Fig. 3.

TABLE III

CAPACITY FACTORS OF DIPEPTIDES ON THE CATION EXCHANGERS STUDIED

Mobile phase, 0.002 *M* potassium dihydrogenphosphate, pH 5.3.

Dipeptide	Capacity factor		
	HEMA Bio 1000 CM	HEMA Bio 1000 SB	Partisil SCX
Gly–Asp	1.09	0.57	0.20
Gly–Glu	1.40	0.86	0.30
Gly–Gly	2.17	2.86	1.00
Gly–Ala	2.20	2.86	1.17
Gly–Val	2.61	4.00	1.60
Gly–Leu	3.28	6.79	2.04
Gly–Phe	4.80	14.4	1.70
Gly–Tyr	5.91	21.86	1.10
Gly–Met	2.52	6.21	1.65

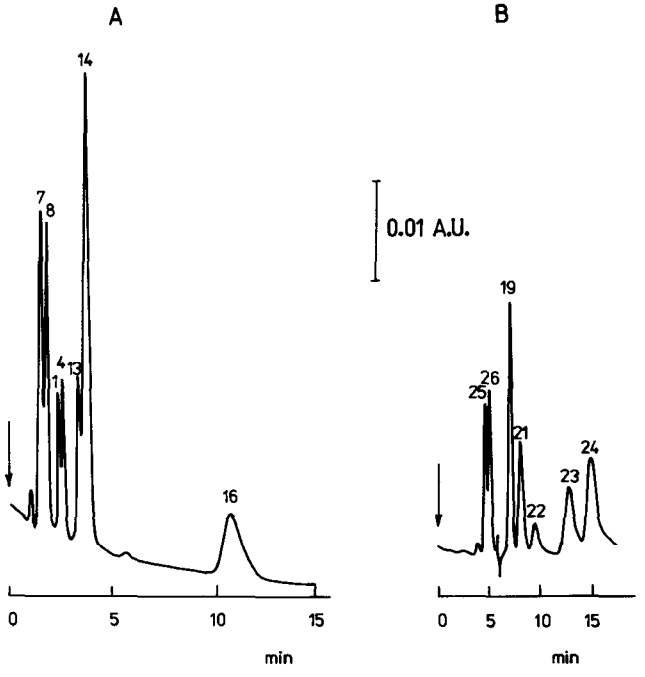


Fig. 5. Chromatogram of selected amino acids (A) and dipeptides (B). HEMA Bio 1000 CM column; mobile phase, 10^{-3} *M* phosphate buffer with 5% (v/v) methanol; UV photometric detection at 210 nm. Peaks: 1 = Gly; 4 = Leu; 7 = Asp; 8 = Glu; 13 = Phe; 14 = Tyr; 16 = Trp; 19 = Gly–Gly; 21 = Gly–Val; 22 = Gly–Leu; 23 = Gly–Phe; 24 = Gly–Tyr; 25 = Gly–Asp; 26 = Gly–Glu.

the HEMA columns cease to be useful and the Partisil column becomes optimal, as demonstrated by Fig. 5 in our previous paper [30].

The effect of the type of the displacer salt cation was studied in detail for the Partisil SCX column in our previous paper [30]. It has been shown that the plots of the log k' values against the ratio of the hydrated ion radius r and its charge number z are linear:

$$\log k' = a(r/z) + b$$

where slope a and intercept b are constants characteristic of the system, and verify the validity of the ion-exchange mechanism. The same dependences were obtained for the HEMA Bio 1000 CM and SB columns in this paper (see also Fig. 3 and Table II in ref. 30).

An example of a separation of amino acids and of dipeptides containing the same amino acid residues is given in Fig. 5. As the UV photometric and amperometric detection was studied in detail in our previous papers [13,14,29,30], it is not discussed here.

It can be concluded that the HEMA phases that combine the ion-exchange, hydrophobic and size-exclusion mechanism widen the possibilities of separation of amino acids and dipeptides. For longer peptides it is preferable to use columns with pure ion-exchange properties.

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