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Anomalous retention behaviour of peptides on porous graphitized carbon column

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Abstract

The retention behaviour of fourteen peptides was determined on a porous graphitized carbon (PGC) column, using acetonitrile–water mixtures as eluents. The majority of peptides showed irregular retention behaviour, their retention decreased with increasing concentration of acetonitrile in the lower concentration range, reached a minimum and increased again with increasing concentration of acetonitrile in the higher concentration range. Both quadratic and bilinear equations described well the irregular retention of peptides on the PGC column, however, the coefficients of correlation were slightly higher for the quadratic function. Calculations indicated that the impact of hydrophobicity, steric and electronic parameters on the retention of peptides on a PGC column is similar, that is a PGC column shows mixed retention mechanisms in which hydrophobic, steric, and electronic forces are equally involved. © 1997 Elsevier Science B.V.

Keywords: Principal component analysis; Porous graphite carbon; Peptides

1. Introduction

Due to the considerable importance of peptides in health care [1], and in biochemical [2] and biophysical research [3] many high-performance liquid chromatographic (HPLC) methods have been developed and applied for their separation and purity control [4]. The overwhelming majority of peptide separations are carried out in the reversed-phase (RP) separation mode [5] using a wide variety of RP supports such as C_2 [6], C_4 [7], C_8 [8] and C_{18} [9] coated silica. Sometimes peptides showed anomalous retention behaviour: their retention decreased with increasing organic component concentration in the lower concentration range, reached a minimum and then increased with further increase in the proportion of organic component. This phenomenon was tentatively explained in terms of a silanophilic effect: at higher organic component concentrations, the solute molecules have an enhanced probability of access to the free silanol groups uncovered by the impregnating agent. The interaction of peptides with the free silanol groups results in an increased retention [10,11]. The relationship between the retention of peptides and the concentration of the organic modifier in the eluent has been successfully described using quadratic functions [12,13].

Porous graphitized carbon (PGC) has been developed as a very insoluble and stable HPLC support [14,15]. It has been successfully used for the separation of diastereomers [16], geometrical isomers [17], and various basic compounds [18] such as tioconazole derivatives. The influence of physicochemical parameters of some ring-substituted aniline derivatives on their retention on PGC column have also been studied. It has been established that the retention on PGC column of ring-substituted aniline

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derivatives is mainly governed by the steric parameters of the substituents [19,20].

Principal component analysis (PCA) [21] has frequently been used in chromatography to extract maximum information from retention data matrices of many dimensions. Thus, PCA has been used in thin-layer chromatography for the classification of flavonoids and chromatographic systems [22]; in HPLC for the characterization of imidazol(in)e [23] and antihistamine drugs [24], and the elucidation of retention mechanisms of PGC support [25] and that of hydrophobic interaction chromatographic media [26]. As the evaluation of the multidimensional matrices of PC loadings and variables is difficult, nonlinear mapping techniques can be used for the reduction of the dimensionality of such matrices [27].

The objectives of this work were to demonstrate the irregular retention behaviour of some peptides on PGC column, to elucidate the effect of various molecular substructures on the retention and to find the relationship between the retention parameters and solute characteristics.

2. Experimental

The PGC column (Shandon Hypercarb 100×4.7 mm I.D., particle diameter 7 μm) was purchased from Shandon Scientific (UK). The HPLC system consisted of a Liquopump Model 312 (Labor MIM, Budapest, Hungary) pump, a Cecil CE-212 variable-wavelength UV detector (Cecil Instr., Cambridge, UK), a Valco injector (Valco, Houston, TX, USA) with a 20 μl sample loop and a Waters 740 integrator (Waters–Millipore, Milford, MA, USA). The flow-rate was 1.0 ml min^{-1} and the detection wavelength was set to 200 nm. Mixtures of acetonitrile–water were used as eluents, acetonitrile concentration ranged from 1.00 to 98.0% (v/v). The eluents were not buffered because the effect of buffer is lower on PGC than on the traditional octadecylsilica column and the irregular retention behaviour of peptides can be also observed in buffered eluent systems. The column was not thermostated: each determination was run at room temperature. The chemical structures of peptides are shown in Table 1. We are well aware that the number of peptides included in the experiment is markedly lower than that used for the study of the retention mechanism of octadecylsilica [28] and PGC [29] supports. As the main objective of the experiment was the demonstration of the irregular retention behaviour of peptide on a PGC column previously described only on octadecylsilica support, we assume that this selection of peptides is suitable for the experimental proof of the irregular retention behaviour. The use of a high number of peptides containing aromatic ring structure was motivated by the fact that solutes with similar ring structure preferably bind to PGC and it was of interest to establish that this stacking interaction occurs also in the case of peptide solutes. To prove this conception the use of basic, acidic or longer peptide solutes will be irrelevant. Peptides were dissolved in the eluents at the concentration of 0.05 mg ml^{-1}. The retention time of peptides in each eluent was determined with three consecutive determinations.

Table 1
Structures of peptides

No. of peptides	Structure
I	H–Gly–Gly–OH
II	H–Glu–Cys–Gly–OH (glutathione reduced)
III	H–Tyr–Phe–OH
IV	H–Tyr–Arg–OH
V	H–Phe–Leu–OH
VI	H–Phe–Glu–OH
VII	H–Phe–Gly–OH
VIII	H–Arg–Phe–OH
IX	H–Phe–Pro–OH
X	H–Phe–Arg–OH
XI	H–Phe–Tyr–OH
XII	H–Tyr–Gly–OH
XIII	Glutathione oxidized
XIV	Polymyxin

Both quadratic (Eq. (1)) and bilinear equations (Eq. (2)) [30] were used for the description of the relationship between the retention of peptides and the acetonitrile concentration in the eluent:

$$\log k' = \log k'_0 + b_1 C + b_2 (C)^2 \quad (1)$$

$$\log k' = \log k'_0 + b_3 \log C - b_4 \log(\beta \cdot 10^C + 1) \quad (2)$$

where k' is the capacity factor of a peptide at a given

acetonitrile concentration, and C is the acetonitrile concentration in the eluent (%, v/v). The inclusion of the bilinear equation in the calculation was driven by the fact that in some instances it described the anomalous retention behaviour in chromatography better than the quadratic equation did [31].

To find the similarities and dissimilarities between the physicochemical parameters and retention characteristics of peptides on PGC column PCA was used. The parameters of Eqs. (1) and (2), the acetonitrile concentration in the eluent causing minimal retention (calculated from both Eqs. (1) and (2)), the hydrophobic, steric and electronic parameters of peptides were the variables and the peptides were the observations. The physicochemical parameters of peptides were calculated according to the data in Ref. [32]. The limit of the variance explained was set to 99.0%. For easier visualization of the results the two-dimensional nonlinear maps of PC loadings and variables were also calculated. The iteration was carried out to the point when the difference between the two last iterations was lower than 10^{-8}.

Calculations were carried out on an IBM AT computer, the software (PCA and nonlinear mapping) was prepared by Dr. Barna Bordás, Plant Protection Institute of Hungarian Academy of Sciences, Budapest, Hungary. The use of this software instead of the Lotus 1-2-3 software was motivated by the fact that this software contains the nonlinear mapping technique too.

3. Results and discussion

The majority of peptides showed irregular retention behaviour, their retention decreased with increasing concentration of acetonitrile in the lower concentration range, reached a minimum and increased again with increasing concentration of acetonitrile in the higher concentration range (Fig. 1). This retention behaviour is highly similar to that observed in the RP-HPLC of peptides in Ref. [10] and explained by the silanophilic effect. However, this explanation is hardly valid in our case, the support being PGC without free silanol groups on the surface. We assume that higher concentrations of acetonitrile suppresses the dissociation of the polar substructures in peptides resulting in increased apparent hydrophobicity. As hydrophobic compounds may attach more strongly to the apolar PGC surface the apparent increase in hydrophobicity may account for the anomalous retention behaviour. However, it cannot be excluded that higher acetonitrile concentration modifies the dimensions and composition of the solvate shell around the peptide molecules increasing in this manner the contact surface between the stationary phase and the solvated solute molecules. According to our knowledge the irregular retention behaviour of peptides on a PGC column has not been previously demonstrated and studied in detail.

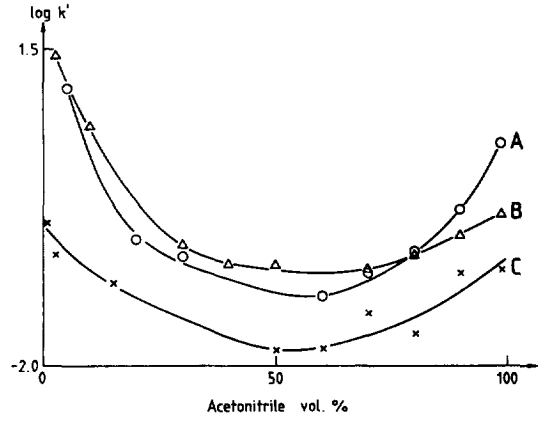

Fig. 1. Relationship between the log k' value of peptides H–Arg–Phe–OH (A), H–Phe–Gly–OH (B) and H–Gly–Gly–OH (C), and the acetonitrile concentration in the eluent.

The parameters of quadratic and bilinear equations describing the relationship between the log k' value of peptides and the acetonitrile concentration in the eluent are compiled in Tables 2 and 3, respectively. The relationship between log k' and organic phase concentration was significant in each instance confirming the applicability of both Eqs. (1) and (2). The parameters differ considerably from each other indicating that the peptides can be easily separated on the PGC column in acetonitrile–water eluent. The ratio of variance explained by Eqs. (1) and (2) varied between 59–98% (quadratic function) and between 69–98% (bilinear function). This finding indicates that both quadratic and bilinear functions are suitable for the description of this type of irregular retention behaviour. As the ratio of variance explained was similar for both equations the advantages of the

Table 2
Parameters of quadratic function describing the relationship between the retention of peptides on porous graphitized carbon column (log k'_0) and the acetonitrile concentration (%, v/v) in the eluent (C): log $k' = \log k'_0 + b_1 C + b_2 (C)^2$

Parameter	No. of peptides						
	I	II	III	IV	V	VI	VII
log k'_0	−0.51	1.46	2.78	1.13	1.48	1.13	1.62
$-b_1 \cdot 10^2$	4.77	9.60	10.81	7.89	7.48	7.58	8.79
$s_{b1} \cdot 10^3$	6.54	8.65	10.54	6.00	6.02	7.21	8.81
$b_2 \cdot 10^4$	4.55	8.70	8.51	6.86	5.86	6.54	7.10
$s_{b2} \cdot 10^5$	6.79	9.02	8.73	5.77	5.88	7.07	8.35
r^2	0.9021	0.9489	0.9478	0.9657	0.9763	0.9613	0.9540
$F_{calc.}$	27.68	74.32	54.48	98.39	123.61	62.06	62.19
	No. of peptides						
	VIII	IX	X	XI	XII	XIII	XIV
log k'_0	1.34	1.86	1.02	2.48	1.35	0.99	−0.21
$-b_1 \cdot 10^2$	9.97	9.69	8.12	9.75	7.29	2.26	0.66
$s_{b1} \cdot 10^3$	9.70	9.89	8.04	9.23	10.62	4.99	1.95
$b_2 \cdot 10^4$	9.25	7.89	7.45	7.65	6.11	n.s.	n.s.
$s_{b2} \cdot 10^5$	9.15	9.12	7.85	7.65	9.82	n.s.	n.s.
r^2	0.9548	0.9545	0.9368	0.9507	0.9162	0.6957	0.5875
$F_{calc.}$	52.87	63.00	51.88	57.89	27.34	20.57	11.39

n.s. = not significant

Table 3
Parameters of bilinear function describing the relationship between the retention of peptides on porous graphitized carbon column (log k'_0) and the acetonitrile concentration (%, v/v) in the eluent (C): log $k' = \log k'_0 + b_3 \log C - b_4 \log (\beta \cdot 10^C + 1)$

Parameter	No. of peptides						
	I	II	III	IV	V	VI	VII
log k'_0	−0.58	1.24	2.51	0.91	1.53	1.29	1.75
$-b_1$	0.28	0.65	0.74	0.50	0.66	0.83	0.89
$s_{b1} \cdot 10^2$	4.3	7.4	10.5	6.1	4.7	9.8	7.1
b_2	0.52	0.88	0.93	0.81	0.69	0.97	0.98
$s_{b2} \cdot 10^2$	8.8	12.2	14.2	11.5	6.2	13.0	9.1
log β	−5.09	−4.17	−4.57	−5.07	−3.58	−3.07	−3.19
r^2	0.8957	0.9233	0.9090	0.9220	0.9835	0.9532	0.9750
$F_{calc.}$	14.32	28.08	16.65	23.66	99.11	27.17	64.93
	No. of peptides						
	VIII	IX	X	XI	XII	XIII	XIV
log k'_0	1.33	1.88	0.94	2.42	2.12	0.96	−0.11
$-b_1 \cdot 10^1$	0.80	0.82	0.61	0.74	1.21	0.22	0.13
$s_{b1} \cdot 10^2$	11.7	7.4	7.5	8.3	13.8	9.4	4.2
b_2	1.21	0.97	0.90	0.90	1.31	n.s.	n.s.
$s_{b2} \cdot 10^2$	18.0	10.0	11.9	10.8	16.1	n.s.	n.s.
log β	−4.27	−3.79	−4.20	−4.37	−2.55	−7.40	−5.29
r^2	0.9216	0.9686	0.9176	0.9417	0.9586	0.6962	0.9214
$F_{calc.}$	15.68	51.48	22.28	26.91	30.97	5.35	3.91

n.s. = not significant.

application of bilinear function instead of quadratic one cannot be established. It has to be emphasized that this conclusion is only an empirical one based on the results of the experiments and its extrapolation to other chromatographic systems may be subject to considerable errors.

The results of PCA are compiled in Table 4 (peptides XIII and XIV were excluded from the calculation because they showed regular retention behaviour). The overwhelming majority of the information contained in the original data matrix can be described by four background variables. In other words, four theoretical variables are sufficient to describe the relationship between the physicochemical parameters and retention characteristics of peptides on PGC column. Unfortunately, PCA does not define these variables as concrete physical or physicochemical entities, only indicates their mathematical possibility. Both retention characteristics and physicochemical parameters have high loadings in the first PC indicating each physicochemical parameter exerts a similar impact on the retention characteristics, that is, hydrophobic and hydrophilic forces are equally involved in the interaction of peptides with the surface of PGC.

The two-dimensional nonlinear map of PC loadings is shown in Fig. 2. The distribution of parameters on the map entirely supports our previous conclusions. The chromatographic characteristics do not form distinct clusters with any of the physicochemical parameters proving again the similar impact of sterical conditions, hydrophobicity and electronic parameters of peptide solutes on the retention on PGC column.

Peptides containing Phe form a cluster on the two-dimensional nonlinear map of PC variables (Fig. 3). The cluster formation suggests that the ring structures of peptides influence not only the strength of the interaction of peptide solutes with the surface

Table 4
Similarities and dissimilarities between the physicochemical parameters and retention characteristics of peptides on porous graphitized carbon column.Results of principal component analysis.

Eigenvalue	Variancee explained (%)	Total variance explained (%)
5.80	48.33	48.33
2.58	23.79	72.12
1.83	15.21	87.34
1.03	8.57	95.91

Principal component loadings

Parameter	No of principal component			
	1	2	3	4
Eq. (1)				
$\log k'_0$	0.92	0.35	−0.04	0.07
b_1	0.79	0.41	0.35	0.29
b_2	0.58	0.35	0.63	0.34
C_{min}	0.74	0.13	−0.62	−0.15
Eq. (2)				
$\log k'_0$	0.98	0.05	−0.09	−0.01
b_1	−0.77	0.61	−0.07	0.04
b_2	0.66	−0.49	0.52	−0.06
$\log \beta$	0.50	−0.81	−0.20	−0.06
C_{min}	−0.32	0.92	−0.12	0.00
Physicochemical parameters				
Hydrophobiity	−0.76	−0.35	0.41	0.32
Bulkiness	0.56	0.43	0.31	−0.55
Electronic properties	0.47	−0.01	−0.56	0.62

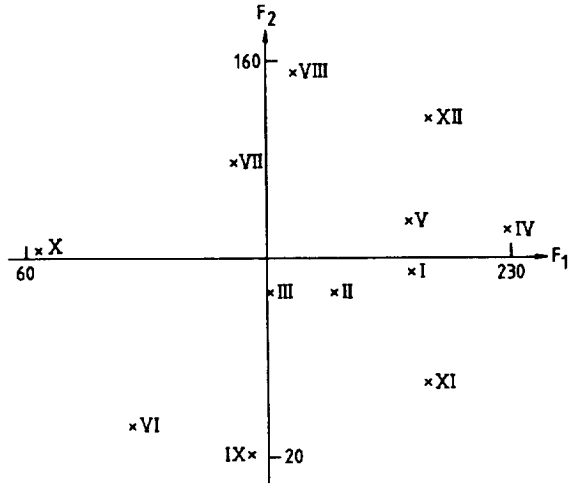

Fig. 2. Relationship between the various physicochemical parameters of peptides and their retention behaviour on porous graphitized carbon column. Two-dimensional nonlinear map of principal component loadings. Number of iterations: 344. Maximum error: $4.18 \cdot 10^{-2}$. I, II and III = $\log k'_0$, b_1 and b_2 values of Eq. (1), respectively; IV and IX = acetonitrile concentrations causing minimal retention calculated by Eqs. (1) and (2), respectively; V, VI, VII and VIII = $\log k'_0$, b_1, b_2 and $\log \beta$ values of Eq. (2), respectively; X, XI and XII = hydrophobicity, sterical and electronic parameters of peptides, respectively.

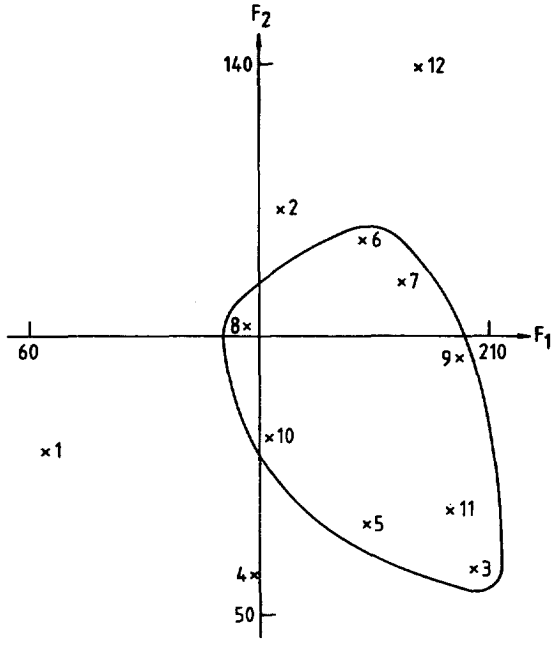

Fig. 3. Distribution of peptides according to their retention behaviour on porous graphitized carbon column. Two-dimensional nonlinear map of principal component variables. Number of iterations: 96. Maximum error: $3.72 \cdot 10^{-2}$. Numbers refer to peptides in Table 1.

of PGC but also the other retention parameters. This effect can be tentatively explained by the assumption that the ring structure of Phe is probably bonded to the hexagonal graphitic substructures on the PGC surface by stacking interactions influencing in this manner the retention behaviour of these derivatives.

It can be concluded from the data that peptides may show irregular retention behaviour on PGC column using acetonitrile–water eluent systems. The retention mechanism underlying this irregular retention behaviour is not well understood. PCA can be successfully used for the elucidation of the relationship between the various physicochemical parameters and retention characteristics. Calculations indicated that the steric parameters of substituents exert the highest impact on retention.

Acknowledgments

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