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# Quantitative relationship between the retention of peptides on a reversed-phase alumina support and their physicochemical parameters

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**Abstract**

The retention time of 16 peptides was determined on polyethylene-coated alumina stationary phase using mixtures of acetonitrile (MeCN) and acidic and basic phosphate buffers as mobile phases. The majority of peptides showed nonlinear retention behaviour, their retention decreased with increasing concentration of MeCN in the lower concentration range, reached a minimum and increased again with increasing concentration of MeCN in the higher concentration range. Quadratic equations described well the atypical retention behaviour of peptides, the significance level being always over 95%. Principal component analysis indicated that the steric parameters of peptides exerted the highest influence on their retention on polyethylene-coated alumina stationary phase. 

*Keywords:* Principal component analysis; Alumina, polyethylene-coated; pH effects; Peptides

## 1. Introduction

Because of its high selectivity and versatility high-performance liquid chromatography (HPLC) has been frequently used for the separation of peptide mixtures [1,2]. A considerable number of HPLC supports have been evaluated for their capacity to separate peptides. Although the use of amino-coated silica support [3], polybutadiene-coated zirconia [4] and micro spherical carbon [5] stationary phases have been reported, the overwhelming majority of peptide separations has been carried out on silica-based reversed-phase supports [6]. However, the application of silica or silica-based reversed-phase supports is limited by the low stability of silica at alkaline pH [7], and the undesirable electrostatic interactions between the polar sub-structures of solutes and the free silanol groups not covered by the hydrophobic ligand [8].

The higher pH stability of alumina makes it a valuable substitute for silica [9]. The retention characteristics of alumina and silica have been previously compared [10]. Alumina support has been applied not only as a stationary phase in adsorption HPLC but also as a basic material for the preparation of various reversed-phase supports [11].

The objectives of the work were the study of the retention behaviour of some peptides on poly-ethylene-coated alumina support, the elucidation of the effect of pH on the retention, and the assessment of the relationship between molecular structure of peptides and their retention behaviour.

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## 2. Materials and methods

Alumina support of 5 μm particle size has been prepared by the research group of Dr. L. Zsembery (Research and Development Laboratory of Hungarian Alumina Trust, Budapest, Hungary). Coating of the alumina support with 5% (w/w) polyethylene (Tiszai Vegyi-kombinát, Hungary) has been carried out in our laboratory as previously described [12]. Columns of 25 cm×4 mm I.D. were filled with a Shandon analytical HPLC packing pump (Pittsburgh, PA, USA) by the procedure proposed for the filling of reversed-phase columns. The HPLC equipment consisted of a Gilson gradient analytical system (Gilson Medical Electronics, Villiers-le-Bel, France) with two piston pumps (Model 302), a detector (Model 116), a Rheodyne injector with 20-μl sample loop (Cotati, CA, USA), and a Waters 740 integrator (Milford, MA, USA). Solvents and buffers were purchased from Merck (Darmstadt, Germany).

Peptides have been synthetized at the Research and Development Laboratory of Reanal (Budapest, Hungary). Acidic mobile phases consisted of mixtures of acetonitrile (MeCN) and aqueous $KH_2PO_4$ buffer (pH 4.5), the concentration of MeCN varying between 10 and 75% (v/v) in steps of 5% (v/v). Basic mobile phases contained MeCN and aqueous $K_2HPO_4$ buffer (pH 8.5), the concentration of MeCN being between 5 and 50% (v/v) in steps of 5% (v/v). The end concentration of the buffer in the mobile phases was 50 m*M* in each instance. The use of different concentrations of MeCN in acidic and basic mobile phases was necessitated by the fact that reasonable retention time of peptides has been obtained in these concentration ranges. The flow-rate was 0.8 ml $min^{-1}$ and the detection wavelength was set at 210 nm. The chemical structures of peptides are shown in Table 1. Peptides have been dissolved in the mobile phases at a concentration of 0.05 mg $ml^{-1}$. The column was not thermostated, each determination has been run at ambient temperature (20±2°C). The retention time of peptides in each eluent was determined with three consecutive determinations and the mean value and the standard deviation of the retention time have been calculated for each peptide in each eluent system. The main objective of the study was the elucidation of the retention behaviour of peptides on a polyethylene-coated alumina ($Alu_{PEE}$) stationary phase and the assessment of the relationship between the retention time and the concentration of MeCN in the mobile phase. As peptides contained neutral, dibasic, dicarboxylic and bulky amino acids it can be assumed that the selection of peptides is suitable for the determination of the character of retention behaviour of peptides on $Alu_{PEE}$ stationary phase. As in the most of the cases the peptides show nonlinear retention behaviour a quadratic equation has been used for the description of the relationship between the retention of peptides and the concentration of MeCN in the mobile phase:

$$\log k = \log k_0 + b_1 C + b_2 C^2 \quad (1)$$

where $k$ is the capacity factor of a peptide at a given concentration of MeCN in both the acidic and basic mobile phases, $k_0$ is capacity factor extrapolated to zero concentration of MeCN in the mobile phase, and $C$ is the concentration of MeCN (%, v/v). The parameters of Eq. (1) have been calculated separately for each peptide in both acidic and basic eluent systems. It has to be emphasized that in the case of regular retention behaviour, the $b_2$ value becomes zero and Eq. (1) transforms to the well known traditional equation generally used to describe retention behaviour:

$$\log k = \log k_0 + b_1 C \quad (2)$$

Table 1
Chemical structures of peptides

| Peptide No. | Structure |
|---|---|
| 1 | H–Phe–Pro–OH |
| 2 | H–Glu–Cys–Gly–OH (glutathione reduced) |
| 3 | Glutathione oxidized |
| 4 | H–Phe–Arg–OH |
| 5 | H–Phe–Leu–OH |
| 6 | H–Tyr–Gly–OH |
| 7 | H–Arg–Phe–OH |
| 8 | H–Phe–Gly–OH |
| 9 | H–Tyr–Ala–OH |
| 10 | H–Tyr–Arg–OH |
| 11 | H–Gly–Gly–OH |
| 12 | H–Phe–Tyr–OH |
| 13 | H–Tyr–Phe–OH |
| 14 | H–Phe–Glu–OH |
| 15 | H–Arg–Gly–Asp–OH |
| 16 | H–Tyr–Gly–Gly–Phe–Met–OH |

Table 2
The hydrophobic ($z_1$), steric ($z_2$) and electronic parameters ($z_3$) of peptides calculated by the additivity rule from the data in Ref. [14]

| Peptide No. | Physicochemical parameters | | |
|---|---|---|---|
| | $z_1$ | $z_2$ | $z_3$ |
| 1 | 3.60 | 2.27 | 4.03 |
| 2 | 2.60 | −5.55 | 0.92 |
| 3 | 5.20 | −11.10 | 1.84 |
| 4 | −0.41 | 4.11 | −2.35 |
| 5 | −7.28 | 0.58 | 0.43 |
| 6 | −1.19 | −2.05 | 0.46 |
| 7 | −0.41 | 4.11 | −2.35 |
| 8 | −1.23 | −2.32 | 0.55 |
| 9 | −2.45 | −0.29 | 1.22 |
| 10 | −6.79 | 4.38 | −3.36 |
| 11 | 4.78 | −8.24 | −0.84 |
| 12 | −7.20 | 3.87 | 0.93 |
| 13 | −7.20 | 3.87 | 0.93 |
| 14 | −2.55 | 1.73 | −0.13 |
| 15 | 7.93 | −1.11 | −2.27 |
| 16 | 5.30 | −4.24 | 0.40 |

Numbers refer to peptides in Table 1.

To find the similarities and dissimilarities between the physicochemical parameters ($z_1$, $z_2$, $z_3$) and retention characteristics of peptides on the $Alu_{PEE}$ stationary phase principal component analysis (PCA) has been employed [13]. The parameters of Eq. (1) (log $k_0$, $b_1$ and $b_2$ measured in acidic and basic mobile phases), the hydrophobic ($z_1$), steric ($z_2$) and electronic parameters ($z_3$) of peptides were the variables (altogether nine variables) and the peptides were the 16 observations. The physicochemical parameters of peptides listed above have been calculated according to additivity rule from the data in Ref. [14], and are compiled in Table 2. These parameters are dimensionless numbers computed from the individual physicochemical parameters of amino acids. PCA calculation was performed on the normalized values and carried out to the point when the principal components explained the 99% of the total variance. As the evaluation of the matrices of perncipal component loadings and variables is difficult their dimensionality has been reduced to two by the nonlinear mapping technique [15].

## 3. Results and discussion

Peptides showed both regular and atypical retention behaviour on $Alu_{PEE}$ support as demonstrated in the case of peptide No. 3 in Fig. 1. The retention (log $k$) of a small number of peptides decreased linearly with increasing concentration of MeCN in the mobile phase. This regular behaviour has been observed only in acidic mobile phases, and only atypical retention behaviour has been found in basic mobile phase systems. The log $k$ value of some peptides decreased steeply at low concentrations of MeCN and slope of the plot log $k$ versus MeCN concentration decreased less sharply with increasing concentration of MeCN, however, the change of the direction of the relationship has not been observed in the concentration range employed. Other peptides showed slightly different retention behaviour: retention decreased with the increasing concentration of MeCN in lower concentration range, reached a minimum and increased again with increasing concentration of MeCN in the range of higher concentrations.

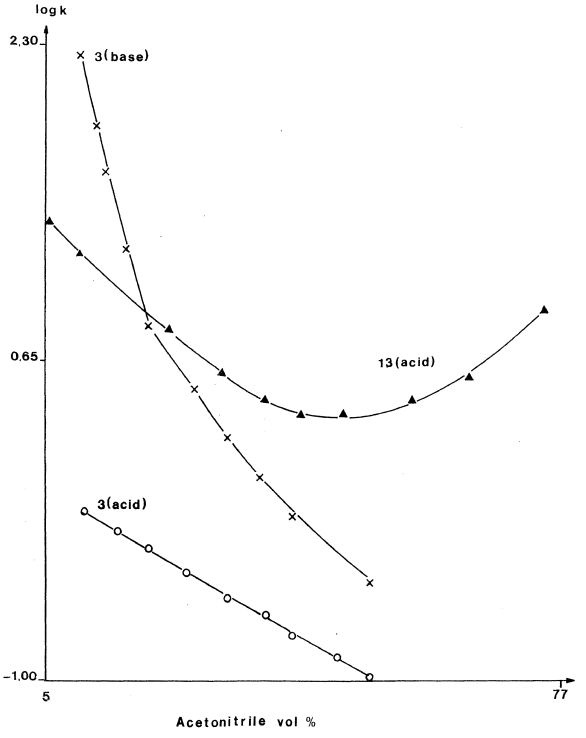


Fig. 1. Relationship between the log $k$ value of peptide Nos. 3 and 13 and the concentration of acetonitrile in acidic and basic mobile phases. Numbers refer to peptides in Table 1.

Table 3
Parameters of quadratic function describing the relationship between the retention of peptides on polyethylene-coated alumina column (log $k$) and the acetonitrile concentration in the acidic eluent ($C$, %, v/v)$\log k = \log k_0 + b_1C + b_2C^2$

| Parameter | No. of peptides | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Log $k_0$ | 1.21 | 0.53 | 0.10 | 1.16 | 1.66 | 0.75 | 1.46 | 0.98 | 1.07 | 1.45 | 0.58 | 1.14 | 1.75 | 1.75 | 0.73 | 1.16 |
| $b_1 \cdot 10^{-2}$ | −3.11 | −1.30 | −2.20 | −4.13 | −3.19 | −5.03 | −4.20 | −4.10 | −5.41 | −5.18 | −2.10 | −6.10 | −5.96 | −3.87 | −2.52 | −2.30 |
| $s_{b1} \cdot 10^{-4}$ | 1.20 | 1.42 | 2.00 | 0.90 | 1.73 | 4.19 | 0.95 | 2.22 | 1.67 | 7.28 | 0.94 | 5.23 | 3.40 | 1.72 | 0.54 | 1.84 |
| $b_2 \cdot 10^{-4}$ | 3.00 | 0 | 0 | 2.71 | 3.85 | 3.71 | 2.97 | 3.33 | 4.41 | 3.51 | 0 | 6.34 | 6.47 | 3.43 | 1.80 | 3.34 |
| $s_{b2} \cdot 10^{-5}$ | 1.86 | 0 | 0 | 1.13 | 0.72 | 2.25 | 1.97 | 2.92 | 2.13 | 1.98 | 0 | 6.13 | 3.45 | 1.73 | 6.71 | 3.38 |
| $r^2$ | 0.9614 | 0.9379 | 0.9325 | 0.9627 | 0.9846 | 0.9627 | 0.9557 | 0.9723 | 0.9445 | 0.9440 | 0.9572 | 0.9772 | 0.9830 | 0.9830 | 0.9894 | 0.9812 |
| $F_{calc.}$ | 53.44 | 50.25 | 51.02 | 51.27 | 54.99 | 51.19 | 52.27 | 56.67 | 54.12 | 54.90 | 51.34 | 54.12 | 53.89 | 53.98 | 58.12 | 47.21 |

Numbers refer to peptides in Table 1.

These retention behaviours can be well described with a quadratic function. Similar retention behaviour has been demonstrated in the reversed-phase HPLC of peptides [16] and it was explained by the silanophilic effect. It means that the peptides have a higher possibility to bind to the free silanol groups on the surface of the silica-based stationary phase not covered by the hydrophobic ligand. However, this explanation cannot be applied for the explanation of this phenomenon, the support being polyethylene-coated alumina without free silanol groups on the surface. However, other free ionisable and polar groups can be present on the surface of this stationary phase. It can be assumed that higher concentrations of MeCN (lower dielectric constant of the mobile phase) suppress the dissociation of the polar substructures in peptides resulting in increased apparent hydrophobicity. As hydrophobic compounds may attach more strongly to the apolar surface of the $Alu_{PEE}$ stationary phase the increase in hydrophobicity caused by the decrease of the dielectric constant of the mobile phase may account for the anomalous retention behaviour. It can be further assumed that the effects observed may be related to ionisation of the analyte or of the alumina surface.

The parameters of equation describing the relationship between the log $k$ value of peptides and the concentration of MeCN in acidic and basic mobile phase are compiled in Tables 3 and 4, respectively. The inclusion of both $r^2$ and $F_{calc.}$ values in Tables 3 and 4 was motivated by the fact that $r^2$ value indicates the ratio of variance explained while $F_{calc.}$ values refer to the significance level of the equation. The zero value of parameter $b_2$ indicates that these peptides show regular retention behaviour in acidic mobile phases. The relationship between the log $k$ and the MeCN concentration was significant at a significance level of 95% in each instance confirming the applicability of Eq. (1) (compare $F_{calc.}$ values with tabulated ones [17]). The ratio of variance

Table 4
Parameters of quadratic function describing the relationship between the retention of peptides on polyethylene-coated alumina column (log $k$) and the acetonitrile concentration in the basic eluent ($C$, %, v/v)$\log k = \log k_0 + b_1C + b_2C^2$

| Parameter | No. of peptides | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Log $k_0$ | 4.33 | 4.87 | 2.42 | 12.18 | 19.19 | 11.71 | 4.66 | 4.66 | 5.52 | 14.38 | 4.44 | 11.68 | 12.85 | 3.06 | 6.44 | 3.12 |
| $b_1 \cdot 10^{-2}$ | −1.61 | −1.34 | −9.45 | −9.97 | −1.74 | −2.43 | −8.75 | −3.42 | −4.27 | −1.00 | −3.78 | −10.00 | −1.18 | −3.25 | −4.62 | −3.79 |
| $s_{b1} \cdot 10^{-4}$ | 0.87 | 9.65 | 4.86 | 0.75 | 0.63 | 1.28 | 4.92 | 1.88 | 2.83 | 2.62 | 1.98 | 5.57 | 1.33 | 3.41 | 1.90 | 2.31 |
| $b_2 \cdot 10^{-4}$ | 9.31 | 4.32 | 7.56 | 6.22 | 1.09 | 1.12 | 5.45 | 1.82 | 2.41 | 6.87 | 1.98 | 6.87 | 1.98 | 6.45 | 7.12 | 2.34 |
| $s_{b2} \cdot 10^{-5}$ | 3.45 | 7.47 | 3.97 | 2.53 | 6.97 | 1.26 | 9.00 | 0.98 | 0.73 | 1.46 | 1.93 | 9.95 | 5.72 | 2.33 | 1.51 | 0.69 |
| $r^2$ | 0.8909 | 0.9034 | 0.9537 | 0.9954 | 0.9902 | 0.8987 | 0.9996 | 0.9878 | 0.9930 | 0.9998 | 0.9920 | 0.9860 | 0.9851 | 0.9063 | 0.8319 | 0.8725 |
| $F_{calc.}$ | 51.21 | 52.70 | 53.74 | 79.85 | 79.11 | 47.13 | 87.27 | 56.21 | 59.63 | 92.17 | 93.01 | 57.24 | 51.42 | 50.23 | 57.23 | 54.12 |

Numbers refer to peptides in Table 1.

explained varied between 83 and 99% (see $r^2$ values). This finding indicates that quadratic function is suitable for the description of the retention behaviour of peptides on an $Alu_{PEE}$ stationary phase. The retention parameters considerably differ from each other indicating that the peptides can be easily separated on a $Alu_{PEE}$ stationary phase using mobile phases appropriately selected. It can be further established that basic media enhance the binding of peptides to the support. This result can be tentatively explained by the supposition that basic pH suppresses the dissociation of the polar substructures of analytes resulting in increased binding to the hydrophobic binding sites on the surface of the stationary phase.

The similarities and dissimilarities among the retention characterstics and physicochemical parameters of peptides calculated by PCA are compiled in Table 5, the parameters having high loadings in the PC components being underlined. The data indicate that the overwhelming majority of the information contained in the original data matrix can be explained by five background (theoretical) components. It means that five theoretical components contain the majority of information held in the nine original variables. Unfortunately, PCA does not define these variables as concrete physical or physicochemical entities, only indicates their mathematical possibility. The most of the retention characteristics and physicochemical parameters have high loadings in the first principal component indicating that these physicochemical parameters can be used for the prediction of the retention behaviour of peptides on $Alu_{PEE}$ stationary phase. However, the second and third principal components indicate that the electronic parameters of peptides also play a considerable role in their retention on $Alu_{PEE}$ surface. The two-dimensional nonlinear map of principal component loadings is shown in Fig. 2. The numbers on the axes do not have any concrete chemical or physicochemical meaning being artefact of the mode of calculation. They only indicate the distribution of the point on a two-dimensional plane where the point 0,0 is irrelevant. The distribution of retention parameters and physicochemical characteristics of peptides indicates that that the steric parameter $z_2$ related to the dimensions of peptides is near to the retention characteristics. This finding suggests that the steric parameters exert the highest influence on the retention and the impact of hydrophobicity is of

Table 5
Differences and similarities between the retention characteristics and physicochemical parameters of peptides on a polyethylene-coated alumina stationary phase

| No. of principal component | Eigenvalue | Variance explained (%) | Total variance explained (%) |
|---|---|---|---|
| 1 | 4.22 | 46.87 | 46.87 |
| 2 | 1.68 | 18.70 | 65.57 |
| 3 | 1.29 | 14.29 | 79.86 |
| 4 | 0.75 | 8.34 | 88.20 |
| 5 | 0.55 | 6.14 | 94.34 |

| Parameter | Principal component loadings<br>No. of principal components | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Log $k_0$ (acidic) | 0.86 | −0.02 | 0.17 | −0.32 |
| $b_1$ (acidic) | 0.88 | 0.12 | −0.13 | 0.28 |
| $b_2$ (acidic) | 0.94 | −0.09 | 0.11 | 0.20 |
| Log $k_0$ (basic) | 0.63 | −0.05 | −0.59 | 0.07 |
| $b_1$ (basic) | −0.09 | 0.78 | 0.03 | 0.56 |
| $b_2$ (basic) | −0.09 | 0.74 | 0.48 | −0.27 |
| $z_1$ | −0.84 | 0.26 | −0.25 | −0.02 |
| $z_2$ | 0.84 | 0.44 | 0.12 | −0.20 |
| $z_3$ | −0.03 | −0.50 | 0.76 | 0.31 |

For symbols see Materials and methods.

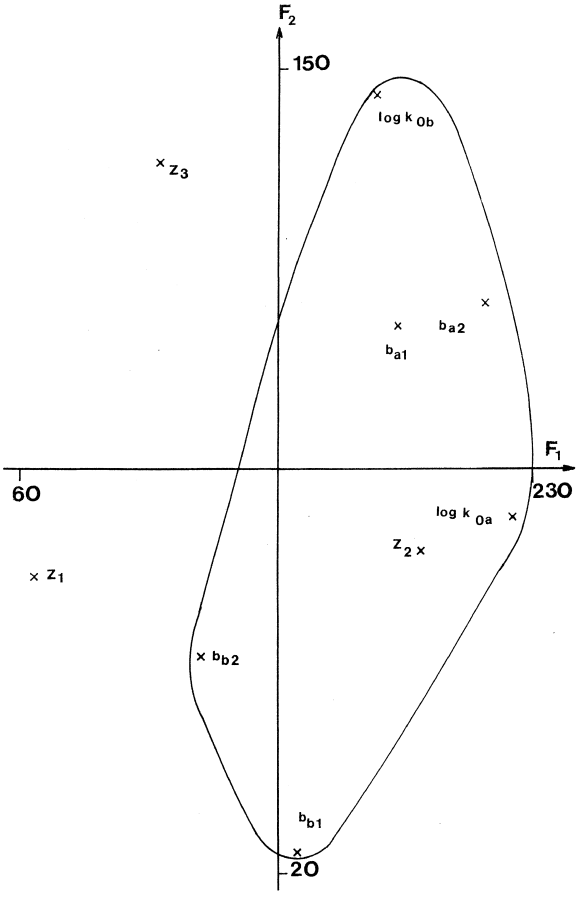


Fig. 2. Relationship between the physicochemical parameters of peptides and their retention behaviour on polyethylene-coated alumina stationary phase. Two-dimensional nonlinear map of principal component loadings. Number of iterations: 103. Maximum error: $3.89 \cdot 10^{-2}$. For symbols see Materials and methods. Subscripts a and b refer to acidic and basic mobile phases, respectively.

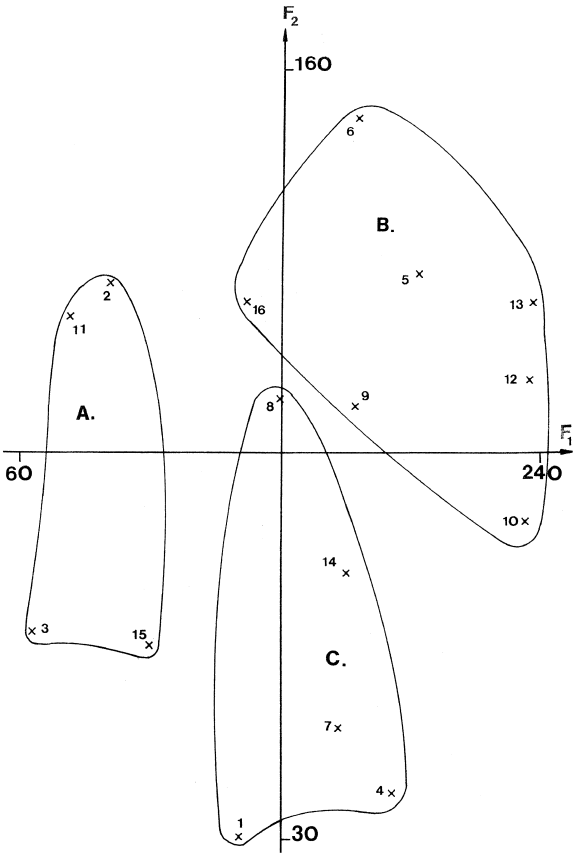


Fig. 3. Distribution of peptides according to their retention behaviour on polyethylene-coated alumina stationary phase. Two-dimensional nonlinear map of principal component variables. Number of iterations: 66. Maximum error: $4.40 \cdot 10^{-2}$. Numbers refer to peptides in Table 1.

secondary importance, however, a reversed-phase chromatographic system has been employed.

The two-dimensional nonlinear map of principal component variables is shown in Fig. 3. The number of iterations for the maps in Figs 2 and 3 is different due to the different matrices of principapl component loadings and variables. The scattering of peptides on the map entirely supports previous conclusions. Peptides without bulky amino acid residues form a clear-cut cluster (cluster A). Except peptide 5, the peptides form clusters according to the presence of tyrosine (cluster B) or phenylalanine residues (cluster C) in the peptide molecule. This finding emphasizes again the predominant role of sterical parameters in the retention of peptides on $Alu_{PEE}$ stationary phase.

## 4. Conclusions

It can be concluded from the data that peptides show a typical retention behaviour on $Alu_{PEE}$ stationary phase in both acidic and alkaline mobile phases the retention decreasing with increasing concentration of MeCN in the mobile phase in the lower concentration range, reaching a minimum, then increasing again at higher concentrations of MeCN.

This retention behaviour has been observed in both acidic and alkaline mobile phases and it has been successfully described by a quadratic function. Calculations has proven that the dimensions of the peptides exert the highest influence on the retention.

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