Binding of Environmental Pollutants to the Wheat Protein Gliadin Studied by High-Performance Liquid Chromatography

Tibor Cserháti*, Esther Forgács, Agnes Kósa, and **Gergely Csiktusnádi Kiss**
Institute of Chemistry, Chemical Research Center, Hungarian Academy of Sciences, P.O. Box 17, 1525 Budapest, Hungary

Roman Kaliszan, Peter Haber, and **Anton Nasal**
Department of Biopharmaceutics and Pharmacodynamics, Medical University, Gdansk, Poland

Ferenc Örsi
Technical University, Department of Biochemistry, Budapest, Hungary

Abstract

The interaction of 23 ring-substituted aniline and 22 ring-substituted phenol derivatives with gliadin is studied by reversed-phase high-performance liquid chromatography. The relationship between the strength of interaction and the physicochemical parameters of the solutes is elucidated by principal component analysis followed by traditional and modified nonlinear mapping. It is established that the sterical parameters of solutes exert the highest influence on the interaction. The impact of polarity parameters is of secondary importance. Nonlinear mapping using the absolute values of principal component loadings explains the interaction more precisely than the traditional nonlinear mapping does.

Introduction

The preparation and application of various protein-coated supports have considerably increased in high-performance liquid chromatography (HPLC). The majority of protein-coated supports were prepared by covalently binding the protein to the support (1). Proteins have generally been bonded to silica (2) or poly-vinylimidazole-coated silica (3). Human serum albumin has been preferentially used for this purpose (4); however, the application of ovoglycoprotein (5), ovomucoid (6), lysozyme (7), and other enzymes has also been reported (8,9). These supports have mainly been used for either the enhancement or the efficacy of chiral separation (10,11). The use of supports prepared by the noncovalent binding of water-insoluble protein (mais protein zein) to silica has also been reported (12).

Principal component analysis (PCA) (13) is a multivariate mathematical–statistical method suitable for the calculation of the

* Author to whom correspondence should be addressed.

Table I. Chemical Structures of Ring-Substituted Aniline Derivatives

	General structure*				
Solute	R_2	R_3	R_4	R_5	R_6
1					
2	CH_3	CH_3			
3	CH_3				CH_3
4	CH_3		CH_3		CH_3
5	OCH_3				
6					OCH_3
7	Cl				
8				Cl	
9			Cl		Cl
10				NO_2	Cl
11	Cl		NO_2		
12	Cl			Cl	Cl
13			Br		
14				Br	
15	Br		Br		
16	Br		Br	Br	
17	I				
18	NO_2				
19			NO_2		
20				NO_2	
21	NO_2				NO_2
22	NO_2				NO_2
23	NO_2			NO_2	NO_2

* R_{2-6} = H except as stated otherwise.

Reproduction (photocopying) of editorial content of this journal is prohibited without publisher's permission.

similarities and dissimilarities between the rows and columns of any data matrix without defining any rows or columns as being the dependent variable. Because of its simplicity, PCA has frequently been used in many fields of chromatography. PCA has been successfully employed for the classification of hydrophobic interactions and hydrophobic interaction chromatographic media (14), the assessment of the retention characteristics of porous graphitized carbon supports (15), and the study of the influence of molecular parameters on HPLC retention of propargylamine derivatives (16). Although PCA reduces the dimensionality of the original data matrix, the resulting matrices of principal component (PC) loadings and PC variables are generally multidimensional. Because the evaluation of the data distributed in multidimensional space is difficult, the dimensions of the matrices of PC loadings and variables can be reduced to 2 by nonlinear mapping techniques (17). Traditional nonlinear mapping takes into consideration the positive or negative signs of the members of the matrices through the construction of the corresponding map. Necessarily, the variables or observations that are strongly but negatively correlated are far away from each other on the map. Unfortunately, the situation is the same when two variables or observations are not intercorrelated: they also are far from each other on the map. When the positive or negative character of the individual regression coefficients is not previously determined, the evaluation of the similarities or dissimilarities among the members of the matrix is subject to error when both negative and positive regression coefficients occur between the members of the original data matrix. This difficulty can be overcome by using the absolute values of regression coefficients for the constructing of the maps.

The objectives of the present study were to determine the binding of ring-substituted phenol and aniline derivatives to the wheat protein gliadin using HPLC, to elucidate the relationship between the strength of binding and the physicochemical parameters of solutes, and to compare the efficacy of PCA combined with traditional and modified nonlinear mapping techniques for this purpose. The study of the binding of phenol and aniline derivatives to gliadin was motivated by the fact that gliadin is an important source of protein in many countries, and phenols are priority pollutants in possible contact with gliadin. The elucidation of the binding may promote not only the better understanding of the interactive forces between proteins and organic pollutants but also may help the development of efficient environmental control procedures.

Table II. Chemical Structures of Ring-Substituted Phenol Derivatives

	General structure*				
Solute	R_2	R_3	R_4	R_5	R_6
24					
25	CH_3				
26			CH_3		
27					CH_3
28					CH_2CH_3
29				CH_3	Cl
30				$N(CH_3)_2$	
31				OCH_3	
32	OCH_3	OCH_3			
33	OCH_3				OCH_3
34			F		
35				Cl	
36				Cl	Cl
37	Cl				Cl
38			Br		
39					Br
40	Br			Br	Br
41					CN
42	NH_2				
43			NH_2		
44					NH_2
45					NO_2

* R_{2-6} = H except as stated otherwise.

Experimental

Gliadin was prepared by the research group of Prof. Ferenc Örsi (Technical University, Department of Biochemistry, Budapest, Hungary). Gliadin-coated silica was prepared by dissolving 0.5

Table III. Mean log k'_w Values of Ring-Substituted Aniline Derivatives on Silica Support Covered by 2.5 and 5% Gliadin*

	log k'_w			log k'_w	
Solute	2.5% gliadin	5% gliadin	Solute	2.5% gliadin	5% gliadin
1	–0.106	–0.011	24	–0.598	–0.319
2	0.123	0.348	25	–0.393	–0.091
3	0.073	0.184	26	–0.398	–0.074
4	0.104	0.379	27	–0.357	–0.053
5	0.270	0.365	28	–0.228	0.010
6	0.436	0.740	29	0.006	0.374
7	0.138	0.315	30	0.177	0.554
8	0.009	0.165	31	–0.453	–0.264
9	0.301	0.750	32	–0.569	–0.211
10	0.048	0.378	33	–0.180	–0.106
11	0.079	0.290	34	–0.470	–0.308
12	0.755	1.188	35	–0.231	0.231
13	–0.015	0.216	36	0.438	1.156
14	0.142	0.464	37	–0.206	0.383
15	0.498	0.911	38	–0.171	0.457
16	1.297	1.665	39	–0.142	0.421
17	0.098	0.380	40	0.533	1.539
18	0.004	0.134	41	–0.598	–0.290
19	–0.131	–0.011	42	–0.290	–0.055
20	–0.194	–0.047	43	–0.523	–0.330
21	0.033	0.290	44	0.029	0.392
22	0.208	0.337	45	–0.584	–0.033
23	0.082	0.294			

* Numbers correspond to solutes in Tables I and II.

and 1 g of gliadin in 200 mL ethanol–water (6:4, v/v) mixtures at 60°C under continuous, gentle stirring. After the dissolution of the protein, 20 g silica (5-µm particle size, Macherey-Nagel, Dürren, Germany) was added and the mixture was stirred for 2 h at the same temperature. Then, the solvents were removed under vacuum. The gliadin-coated silicas with 2.5 and 5% gliadin coating were dried in a vacuum oven at 60°C. A column (250 × 4-mm i.d.) was filled with a Shandon (Pittsburgh, PA) analytical pump using bidistilled water as a filling agent. The HPLC system consisted of a Liquopump model 312 pump (Labor MIM, Budapest, Hungary), a Cecil CE-212 variable wavelength ultraviolet detector (Cecil Instruments, Cambridge, U.K.), a Valco

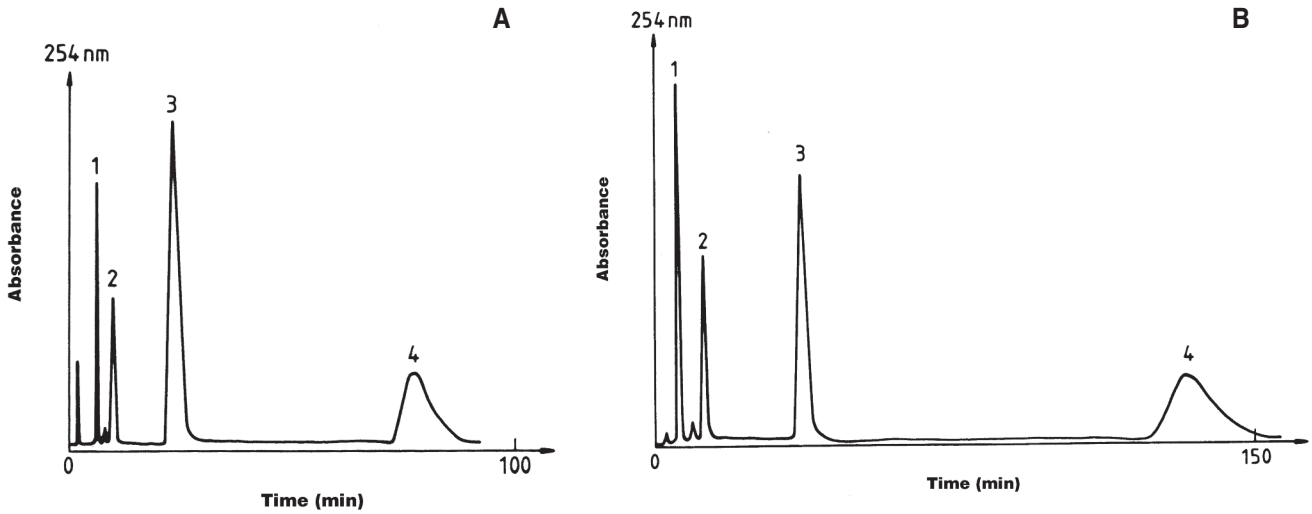

Figure 1. Separation of aniline, 4-bromoaniline, 2,4- dibromoaniline, and 2,4,6-tribromoaniline on gliadin-coated silica columns. Columns were silica coated with 2.5% (w/w) gliadin (A) and silica coated with 5% (w/w) gliadin (B). Conditions: flow rate, 1 mL/min for 55 min, then to 1.5 mL/min; detection wavelength, 254 nm; mobile phase, bidistilled water. Peaks: 1, aniline; 2, 4-bromoaniline; 3, 2,4-dibromoaniline; and 4, 2,4,6-tribromoaniline.

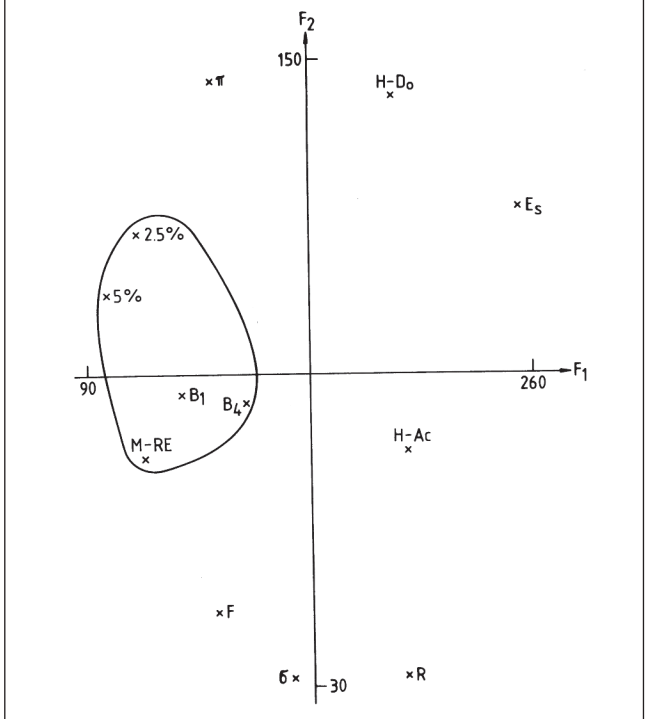

Figure 2. Two-dimensional nonlinear map of principal component loadings calculated form the original matrix, showing the similarities and dissimilarities between the retention characteristics and physicochemical parameters of ring-substituted aniline and phenol derivatives. The number of iterations was 378; maximal error was $4.52 \cdot 10^{-2}$.

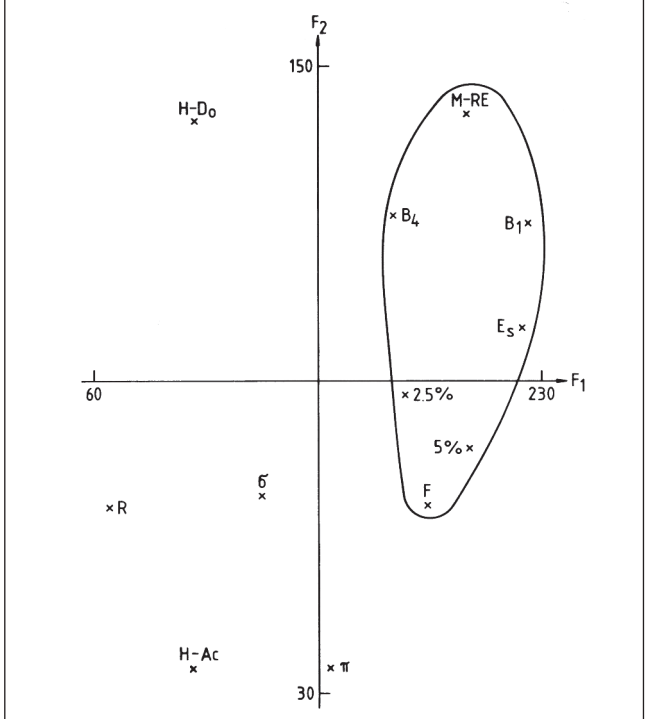

Figure 3. Two-dimensional nonlinear map of principal component loadings calculated form the absolute values, showing the similarities and dissimilarities between the retention characteristics and physicochemical parameters of ring-substituted aniline and phenol derivatives. The number of iterations was 61; maximal error was $1.54 \cdot 10^{-2}$.

(Houston, TX) injector with a 20-µL sample loop and a Waters 740 integrator (Waters-Millipore Inc., Milford, MA). The flow rate was 1 mL/min, and the detection wavelength was 254 nm. Bidistilled water was used as the eluent. The determinations were run at ambient temperature (22–24°C). The chemical structures of ring-substituted aniline and phenol derivatives are compiled in Tables I and II, respectively. The solutes were dissolved in bidistilled water at a concentration of 0.2 mg/mL. Each retention time was determined by 3 consecutive injections. The dead volume of the system was measured by injecting 1% aqueous $NaNO_3$ solution. The log k'_w values and the standard deviation were calculated. It was supposed that a higher log k'_w value indicated a higher affinity of the environmental pollutants to gliadin, and therefore, it can be used as a quantitative indicator of the strengh of solute–protein interaction. To find the correlation between the strength of interaction and the physicochemical parameters of ring-substituted phenol and aniline derivatives, PCA was applied. The log k'_w values and the physicochemical parameters of solutes were the variables, and the solutes were the observations. The physicochemical parameters were as follows: π was Hansch–Fujita's substituent constant characterizing hydrophobicity; H–Ac and H–Do were indicator variables for proton acceptor and proton donor properties, respectively; M–RE was molar refractivity; F and R were electronic parameters characterizing the inductive and resonance effect, respectively; σ was Hammett's constant, characterizing the electron-withdrawing power of the substituent; Es was Taft's constant, characterizing the steric effects of the substituent; and B_1 and B_4 were Sterimol width parameters determined by the distance of substituents at their maximum point perpendicular to attachment (18). These parameters have been previously used in various fields of chromatography: hydrophobicity for the characterization of hydrophobic interaction and hydrophobic interaction chromatography media (14); molar refractivity for the study of the relationship between solute structure and retention on polybuta- diene-coated alumina (19); Taft's constant for the evaluation of selectivity in gas-liquid chromatogaphy (GLC) (20); and various electronic and steric parameters for the elucidation of structure-retention relationship in GLC (21,22), HPLC (23), etc. The ratio of variance explained by PCA was set to 99%. The dimensionality of the resulting matrices of PC loadings and variables was reduced to 2 by the nonlinear mapping technique. The iteration of the nonlinear map was carried out to the point when the difference between the last 2 iterations was lower than 10^{-8}. Because the matrix of PC loadings also contained negative values, nonlinear mapping was also performed using the absolute values of PC loadings.

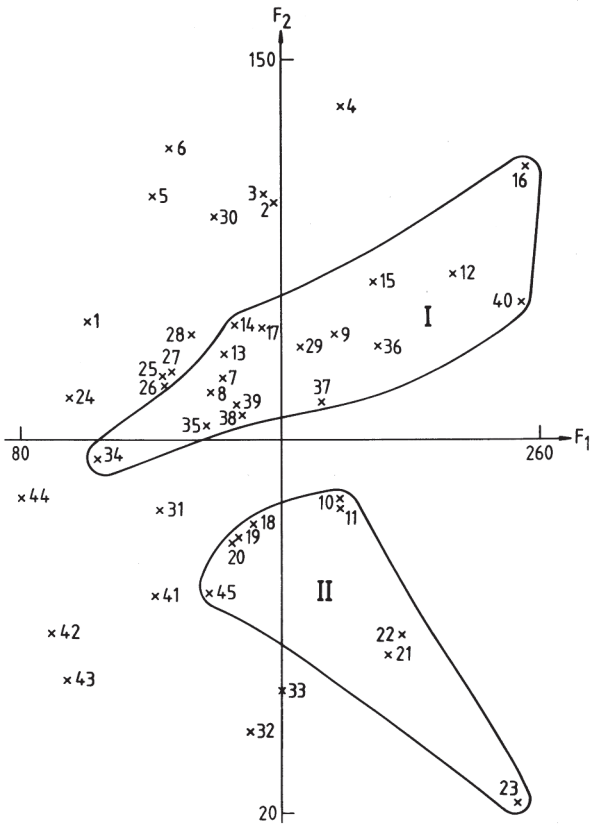

Figure 4. Two-dimensional nonlinear map of principal component variables, showing the similarities and dissimilarities between the ring-substituted aniline and phenol derivatives. The number of iterations was 184; maximal error was $3.35 \cdot 10^{-2}$. The numbers correspond with the solutes in Tables I and II.

Table IV. Similarities and Dissimilarities Between the Physicochemical Parameters of Ring-Substituted Aniline and Phenol Derivatives and their Retention of Gliadin-coated Silica Columns

Results of principal component analysis

Component	Eigen value	Variance explained (%)	Sum of variance explained (%)
1	5.53	46.10	46.10
2	2.60	21.65	67.75
3	1.59	13.26	81.01
4	0.90	7.46	88.47
5	0.62	5.17	93.64

Principal component loadings

Parameters	Principal component 1	2	3	4	5
log k'_w(2.5%)	0.76	–0.39	0.09	0.20	–0.42
log k'_w(5%)	0.79	–0.44	0.01	0.31	–0.17
p	0.46	–0.71	–0.34	–0.01	0.38
H–Ac	0.12	0.80	0.49	–0.22	–0.07
H–Do	–0.32	0.10	0.64	0.61	0.03
M–RE	0.94	–0.02	0.18	–0.12	–0.05
F	0.72	0.50	–0.12	0.21	0.23
R	0.02	0.56	–0.72	0.02	–0.34
s	0.38	0.68	–0.35	0.40	0.24
Es	–0.90	–0.20	0.06	0.10	0.10
B_1	0.96	–0.03	0.09	0.01	0.07
B_4	0.84	0.16	0.33	–0.33	0.14

Results and Discussion

The separation of aniline, 4-bromoaniline, 2,4-dibromoaniline, and 2,4,6-tribromoaniline on the gliadin-coated silica columns is shown in Figure 1. Aniline derivatives are well separated on both columns, and the peaks are symmetric, even at high elution times. Silica support with a higher concentration of gliadin on the surface retains the solutes more strongly, suggesting that the retention is really influenced by the presence of the gliadin layer. Derivatives with more substituents are eluted later on both supports, indicating the influence of steric parameters on the retention.

The mean log k'_w values of ring substituted aniline and phenol derivatives are compiled in Table III. The relative standard deviation was lower than 1.5% in each instance, showing the good stability of the support and good reproducibility of the HPLC system. The data in Table III indicate that the retention of the solutes on the gliadin-coated silica column shows considerable variation. Because the retention in water is related to the strength of gliadin–drug interaction, the differences in log k'_w suggest that the strength of interaction depends on the chemical structure of the solutes.

The results of PCA are summarized in Table IV. Five principal components explain the majority of variance, indicating that the 12 original variables can be substituted by 5 background (abstract) variables with only 6.36% loss of information. Unfortunately, PCA does not prove the existence of such background variables as concrete physicochemical entities, but instead only indicates their mathematical possibility. The log k'_w values, together with the sterical parameters, have a high loading in the first PC, indicating the marked influence of these physicochemical parameters on the strength of gliadin–solute interaction. Interestingly, the hydrophobicity of solutes has a low loading in the first PC, suggesting that the role of apolar, hydrophobic forces is negligible in the protein–solute interaction.

The two-dimensional nonlinear maps calculated from the original PC loadings and from the absolute values of PC loadings are shown in Figures 2 and 3. The log k'_w values determined on silica supports coated with 2.5 and 5% gliadin are very near to each other on both maps, indicating that the gliadin loading has a negligible effect on the selectivity of the support. The maps show marked differences in the distribution of variables, indicating the considerable impact of the modification of the mode of calculation. The Taft's constant characterizing the steric effects of the substituent is far away on the map from log k'_w values calculated from the original PC loadings. It can be concluded, erroneously, that this physicochemical parameter does not influence the strength of the binding of solutes to gliadin. However, the data in Table IV clearly show that the relationship between the log k'_w values and Taft's constant is strong but negative. This finding supports our previous theoretical conclusions that the information contained in the two-dimensional nonlinear map may be misleading when both negative and positive correlations occur between the variables. The distribution of variables on the map calculated from the absolute values (Figure 3) corresponds to the data in Table V. Physicochemical parameters exerting a considerable impact on the strength of gliadin–solute interaction are near the log k'_w values (see cluster) while the other parameters are well separated.

The distribution of ring-substituted aniline and phenol derivatives on the two-dimensional nonlinear map of principal component variables entirely supports our previous conclusions. The solutes form distinct clusters according to the presence of bulky halogen (cluster I in Figure 4) or nitro groups (cluster II in Figure 4) independently of the fact that the solutes are aniline or phenol derivatives. This finding indicates that the importance of the highly polar OH or NH_2 groups in the strength of interaction is relatively low.

Conclusion

It can be concluded from the data that the binding of ring-substituted aniline and phenol derivatives to gliadin can be successfully studied by reversed-phase HPLC. The strength of interaction mainly depends on the sterical parameters of the solutes, and the role of hyrophobic forces is negligible. The use of the absolute values of principal component loadings and variables for the calculation of two-dimensional nonlinear maps prevents the occurrence of errors originating from the positive and negative character of the relationships between the members of the corresponding matrices. Therefore, its application is highly recommended.

Acknowledgments

This work was supported by the Polish-Hungarian cooperation grant "Development of new analytical and evaluation methods for the assessment of biological effect of xenobiotics and pharmaceuticals".

References

1. M.C. Millot, B. Sébille and C. Mangin. Enantiomeric properties of human albumin immobilized on porous silica supports coated with polymethacryloyl chloride. *J. Chromatogr. A* **776:** 37–44 (1997).
2. H. Gao and R.K. Gilpin. Studies of the influence of hydrogen chloride on the liquid chromatographic properties of silica-immobilized bovine serum albumin under normal-phase conditions. *J. Liq. Chromatogr. Relat. Technol.* **20:** 21–29 (1997).
3. N.L. Taleb, M.C. Millot, and B. Sébille. Enantioselectivity properties of human serum albumin immobilized on anion-exchangers based on polyvinylimidazole-coated silica. Effect of protein loading on separation properties. *J. Chromatogr. A* **776:** 45–52 (1997).
4. J. Yang and D.S. Hage. Effect of mobile phase composition on the binding kinetics of chiral solutes on a protein-based high-performance liquid chromatography column: interactions of D- and L-tryptophan with immobilized human serum albumin. *J. Chromatogr. A* **766:** 15–25 (1997).
5. J. Haginaka and H. Takehira. Separation of enantiomers on a chiral stationary phase based on ovoglycoprotein. I. Influences of the pore size of base silica materials and bound protein amounts on chiral resolution. *J. Chromatogr. A* **773:** 85–91 (1997).

6. J. Haginaka and H. Takehira. Separation of enantiomers on a chiral stationary phase based on ovoglycoprotein. II. Comparison of chiral recognition properties with crude ocomucoid. *J. Chromatogr. A* **777:** 241–47 (1997).
7. W. Su, R.B. Gregory, and R.K. Gilpin. Liquid chromatographic studies of silica-immobilized HEW lysozyme. *J. Chromatogr. Sci.* **31:** 285–90 (1993).
8. T. Alebic-Kolbah and I.W. Wainer. Application of an enzyme-based stationary phase to the determination of enzyme kinetic constants and typer of inhibition. New high performance liquid chromatographic approach utilizing an immobilized artificial-membrane chromatographic support. *J. Chromatogr.* **653:** 122–29 (1993).
9. T. Alebic-Kolbah and I.W. Wainer. Enzyme-based high-performance liquid chromatography stationary phases as metabolic reactors. Immobilization of non-solubilized rat liver microsomes on an immobilized artificial membrane high-performance liquid chromatography support. *J. Chromatogr.* **646:** 289–95 (1993).
10. H. Hofstetter, O. Hofstetter, and V. Schurig. Rapid separation of enantiomers in perfusion chromatography using a protein chiral stationary phase. *J. Chromatogr. A* **764:** 35–41 (1997).
11. J. Haginaka and N. Kawasugi. Enantioselectivity of bovine serum albumin-bonded columns produced with isolated protein fragments. II. Characterization of protein fragments and chiral binding sites. *J. Chromatogr. A* **769:** 215–23 (1997).
12. Z. Szikszay and E. Forgács. Study of peptide-protein interaction by high-performance liquid chromatography. Effect of hydrophobic and steric parameters. *Biochem. Mol. Biol. Int.* **42:** 677–84 (1997).
13. M. Turowski, R. Kaliszan, C. Lüllmann, H.G. Genieser, and B. Jastorff. New stationary phases for the high-performance liquid chromatographic separation of nucleosides and cyclic nucleosides. *J. Chromatogr. A* **728:** 201–211 (1996).
14. P. Karsnas and T. Lindblom. Characterization of hydrophobic interaction and hydrophobic interaction chromatography media by multivariate analysis. *J. Chromatogr.* **599:** 131–36 (1992).
15. R. Kaliszan, K. Osmialowski, B.J. Bassler, and R.A. Hartwick. Mechanism of retention in high-performance liquid chromatography on porous graphitized carbon as revealed by principal component analysis of structural descriptors of solutes. *J. Chromatogr.* **499:** 333–44 (1990).
16. T. Cserháti. Evaluation of HPLC retention data by non-linear mapping, cluster analysis and varimax rotation. A comparative study. *J. Biochem. Biophys. Meth.* **32:** 137–50 (1996).
17. J.W. Sammon, Jr. A nonlinear mapping for data structure analysis. *IEEE Trans. Comput.* **C18:** 401–407 (1969).
18. R. Franke. QSAR parameters. In *QSAR and Strategies in the Design of Bioactive Compounds*, J.K. Seydel, Ed. VCH Verlagsgesellschaft, Wienheim, Germany, 1985, pp 59–67.
19. R. Kaliszan and K. Osmialowski. Correlation between chemical structure of non-congeneric solutes and their retention on polybutadien-coated alumina. *J. Chromatogr.* **506:** 3–16 (1990).
20. K. Szymoniak and J.R. Chretien. Factor analysis of trends in selectivity in gas-liquid chromatography of 175 saturated and unsaturated esters on 14 polysiloxane stationary phases. *J. Chromatogr.* **404:** 11–22 (1987).
21. T.F. Woloszyn and P.C. Jurs. Quantitative structure-retention relationship studies of sulfur vesicants. *Anal. Chem.* **64:** 3059–63 (1992).
22. O. Mekenyan, N. Dimov, and V. Enchev. Methodology for deriving quantitative structure-retention relationships in gas chromatography. *Anal. Chim. Acta* **260:** 69–74 (1992).
23. P. Jandera. Correlation of retention and selectivity of separation in reversed-phase high-performance liquid chromatography with interaction indices and with lipophilic and polar structural indices. *J. Chromatogr. A* **656:** 437–67 (1993).

Manuscript accepted March 31, 1999.