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Binding of substituted phenol and aniline derivatives to the corn protein zein studied by high-performance liquid chromatography

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Abstract

The interaction of 12 substituted phenol, three aminophenol and four substituted aniline derivatives with the corn protein zein was studied on zein-coated silica and alumina stationary phases by high-performance liquid chromatography using bidistilled water as mobile phase. Solutes were eluted from the zein-coated supports with different retention times indicating that they bind to the protein with different forces. They were more strongly retained on silica-based than on alumina-based support proving that the original adsorptive character of the support remains even after impregnation. The retention of solutes on both zein-coated stationary phases significantly depended on the steric and electronic parameters of solutes and was independent of the calculated and measured lipophilicity parameters, indicating that hydrophobic forces are not included in the interaction of zein with these class of solutes. It has been concluded that the interaction is governed by steric and electrostatic forces. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Zein; Phenol; Aniline

1. Introduction

Various liquid chromatographic methods such as thin-layer chromatography (TLC) [1] and high-performance liquid chromatography (HPLC) [2,3] have been frequently employed for the study of the interaction between bioactive compounds. Earlier results in the application of chromatographic techniques for the determination of molecular interactions have been previously reviewed [4]. In the last few decades the number of studies dealing with the elucidation of the correlation between molecular structure and chromatographic retention (quantitative structure–retention relationship, QSRR) [5] and between the physicochemical parameters of compounds and the strength of their interaction determined by chromatography [6] considerably increased. Principal component analysis (PCA) [7], a multivariate mathematical–statistical method has been used many times for the evaluation of chromatographic data. PCA calculates the relationships among the columns and rows of any multidimensional matrix without one of them being the dependent variable, and reduces the number of variables by creating theoretical background variables which explain the overwhelming majority of variance in the matrix. PCA was employed in chromatography to study the retention behavior of barbiturates in micellar electrokinetic chromatography [8], to determine the importance of solute ‘‘polarizability corrections’’ in QSRRs [9], to assess the retention characteristics of porous graphitized carbon supports [10], and to the chromato-

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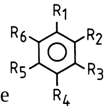
Table 1
Chemical structures of ring-substituted phenol and aniline derivatives

Compound No.	General structure 					
	R_1	R_2	R_3	R_4	R_5	R_6
1	OH	H	H	H	H	H
2	OH	H	H	OH	H	H
3	OH	H	OH	H	H	H
4	OH	H	OCH_3	H	H	H
5	OH	CH_3	H	H	H	H
6	OH	H	CH_3	H	H	H
7	OH	H	H	CH_3	H	H
8	OH	H	H	CN	H	H
9	OH	H	H	Br	H	H
10	OH	H	H	Cl	H	H
11	OH	H	F	H	H	H
12	CHO	H	OH	OH	H	H
13	OH	NH_2	H	H	H	H
14	OH	H	NH_2	H	H	H
15	OH	H	H	NH_2	H	H
16	NH_2	H	H	H	H	H
17	NH_2	NO_2	H	H	H	H
18	NH_2	OCH_3	H	H	H	H
19	NH_2	H	H	OCH_3	H	H

graphic quality control of food products [11,12]. As the evaluation of the resulting multidimensional matrices of PC loadings and variables is difficult the dimensionality of matrices can be reduced by the nonlinear mapping technique [13]. PC loadings are similar to the regression coefficients in the traditional linear regression analysis they indicate the relative weight of the individual variables in the given principal component.

Lipophilicity is an important molecular parameter influencing the retention of solute in reversed-phase separation mode [14] and many interactions between bioactive compounds [15]. Reversed-phase thin-layer chromatography (RPTLC) has been extensively used for the determination of molecular lipophilicity [16]. The advantages of the measurement of lipophilicity by RPTLC are the rapidity, the small quantity of compounds needed for the determination and the less strict requirement of purity because the impurities are separated during the chromatographic process. The lipophilicity values (R_M) determined by RPTLC are generally well correlated with the log *P* values measured by the traditional partition between water and *n*-octanol. The log *P* value is defined as the logarithm of the ratio of concentrations of the analyte distributed between water and *n*-octanol [17]. As the majority of compounds show negligible mobility in RPTLC using water as mobile phase, the elution strength has to be enhanced by adding an organic solvent miscible with water. In order to increase the reliability of the lipophilicity determination, linear relationship is calculated between the concentration of the organic modifier in the mobile phase and the actual R_M value of the molecule. The intercept value of the equation (R_M value extrapolated to zero concentration of organic modifier) is considered as the best estimate of lipophilicity (R_{M0}) [18].

The objectives of the present work were the determination of the binding of ring-substituted phenol and aniline derivatives to the maize protein zein using HPLC, the measurement of the lipophilicity and specific hydrophobic surface of the compounds and the elucidation of the relationship between the capacity to bind to zein and the measured and calculated physicochemical parameters by PCA. The study of the binding of phenol and aniline derivatives to zein was motivated by the fact the zein is a vital protein source in many countries, and phenol and aniline derivatives are priority pollutants in possible contact with zein. The elucidation of the interaction may help not only the more profound understanding of the binding forces between organic pollutants and proteins but also may promote the development of more competent environmental control procedures.

2. Experimental

Zein-coated silica and alumina stationary phases were prepared by dissolving 0.5 g of zein in 200 ml of 1-propanol–water (7:3, v/v) at 70°C under continuous gentle stirring. After the dissolution of the protein 20 g silica (particle size 5 μm, Macherey-Nagel, Dürren, Germany) or alumina (particle size 5 μm, Research Institute of the Hungarian Alumina Trust, Budapest, Hungary) were added and the mixtures were stirred for 2 h at the same temperature. After 2 h the solvent was removed under vacuum in a rotating evaporator. Zein-coated supports were dried in a vacuum oven at 70°C. Columns of 150×4 mm I.D. were filled with a Shandon (Pittsburgh, PA, USA) analytical pump using water as filling agent.

The HPLC system consisted of a Merck–Hitachi (L-6000A) pump, a Merck–Hitatchi (L-4000A) variable-wavelength UV detector, a Rheodyne (CA, USA) injector with a 20-μl sample loop and a Merck–Hitatchi (D-2500A) integrator. The eluent was bidistilled water. The flow-rate was 0.5 ml/min and the detection wavelength was set to 254 nm. The chemical structures of the environmental pollutants are compiled in Table 1. They were dissolved in bidistilled water at a concentration of 0.5 mg/ml. The columns were not thermostated, separations were performed at ambient temperature (20–22°C). The retention time of each compound on both columns was determined with three consecutive determinations. The dead volume of the system was measured by injecting a 1% aqueous $NaNO_3$ solution. The average of the logarithm of the capacity factor (log k'_w) and its relative standard deviation (RSD) were calculated. It was assumed that a stronger retention (higher log k'_w value) indicates a higher affinity of the pollutants to bind to zein, therefore, it can be employed as a quantitative indicator of the strength of solute–protein interaction.

In order to determine the lipophilicity and the specific hydrophobic surface area of aniline and phenol derivatives RPTLC was employed. Polygram SIL G/UV$_{254}$ plates (Macherey-Nagel) and DC-Fertigplatten Aluminiumoxid 60 F$_{254}$ (Merck, Dürren, Germany) were impregnated with *n*-hexane–paraffin oil (97.5:2.5 and 90:10, v/v) by overnight predevelopment. The employment of two different supports and two different degree of hydrophobic coatings was motivated by the findings that reversed-phase stationary phases partially retain their original retention characteristics even after coating and this effect decreases with increasing concentration of hydrophobic ligands on the surface of support. Mobile phases were methanol–water mixtures, methanol concentration varying between 0 and 25% (v/v) in steps of 2.5% (v/v). After development the plates were dried at room temperature, and the spots were detected under UV light. Each determination was run in quadruplicate. The R_M values were calculated by $R_M = \log(1/R_F - 1)$. Linear correlations were calculated between the R_M values of the compounds and the concentration of organic modifier in the eluent:

$$R_M = R_{M0} + bC \qquad (1)$$

where R_M is the actual R_M value of compound determined at a concentration C of organic modifier in the eluent. R_{M0} (intercept) is the R_M value of a compound extrapolated to zero organic phase concentration in the eluent and b (slope) is the lipophilicity change of a compound caused by unit concentration change of the organic phase. The R_{M0} and b values in Eq. (1) were considered to be the best indicator of the lipophilicity and specific hydrophobic surface area of compounds, respectively. The calculation was carried out separately for each compound and for each eluent system.

To find the relationship between the capacity of aniline and phenol derivatives to bind to zein and their measured and calculated physicochemical parameters PCA was applied. The variables were the strength of interaction between zein-coated silica and alumina characterized by the corresponding log k'_w values (log k'_{Sizein} and log k'_{Alzein}), the lipophilicity values and specific hydrophobic surface areas determined on silica-based stationary phases impregnated with 2.5 and 10% paraffin oil ($R_{M0Si2.5}$, $b_{Si2.5}$, R_{M0Si10} and b_{Si10}, respectively), the lipophilicity values and specific hydrophobic surface areas determined on alumina-based stationary phases impregnated identically ($R_{M0AL2.5}$, $b_{Al2.5}$, R_{M0Al10} and b_{Al10}, respectively), and the following calculated physicochemical parameters: π = Hansch–Fujita's substituent constant characterizing hydrophobicity [19,20]. Substituent constant is defined as the difference in octanol–water partition of a compound containing the substituent and of the parent molecule without the given substituent. H-Ac and H-Do = indicator variables for proton acceptor and proton donor properties, respectively [21]. As at the present time a set of hydrogen bonding parameters is not available, a value of 1 was assigned to both hydrogen bond donor and hydrogen bond acceptor substituents and 0 other substituents. M-RE = molar refractivity [22]. Molar refractivity is related to London dispersion forces (polarizability) and can be calculated from the molecular mass, index of refraction and density. F and R = electronic parameters characterizing the inductive and resonance effect, respectively [23]. The inductive constant was defined as the differences in the rate constant for the hydrolysis of substituted and unsubstituted esters and related to the electrostatic effect of substituent on reaction center. Resonance effect is important with strong electron-attracting groups when substituents are directly conjugated with the reaction center. $\sigma =$ Hammett's constant, characterizing the electron-withdrawing power of the substituent [24]. The Hammett constant has been defined as the difference between the ionization constant of benzoic acid in water at 25°C and the ionization constant of a *meta* or *para* substituted benzoic acid derivative under the same experimental conditions. Es = Taft's constant, characterizing steric effects of the substituent [25], it is linearly related to the average of the minimum and maximum van der Waals radii of the substituent. B_1 and B_4 = Sterimol width parameters determined by distance of substituents at their maximum point perpendicular to attachment bond axis L [26,27]. The length of substituent (L) is defined along the axis of the bond between the first atom of the substituent and the parent molecule. The physicochemical parameters of analytes were calculated by the additivity rule. The observations were the ring-substituted phenol and aniline derivatives listed in Table 1. When the hydrophobicity parameters cannot be measured in each TLC system the solute was omitted from the calculations. In order to facilitate the evaluation of the multidimensional matrices of principal component loadings and variables their dimensionality was reduced to two by the nonlinear mapping technique. The iteration of the nonlinear map was carried out to the point when the difference between the last two iterations was lower than 10^{-8}.

Software for PCA and nonlinear mapping was

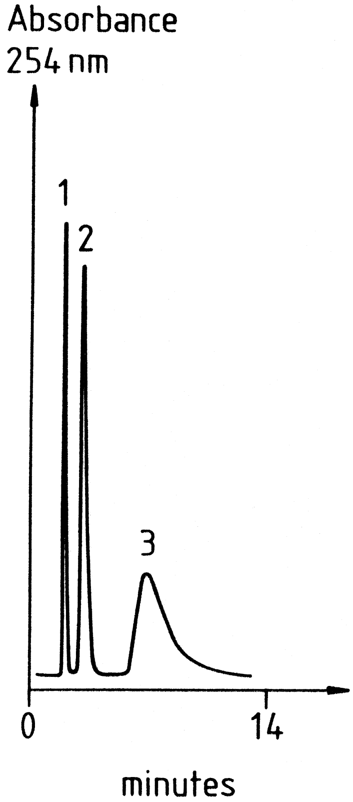


Fig. 1. Separation of 3-aminophenol (1), 3-hydroxyphenol (2) and 3-fluorophenol (3) on a zein-coated silica column. Mobile phase, bidistilled water; flow-rate, 0.5 ml/min; detection wavelength, 254 nm.

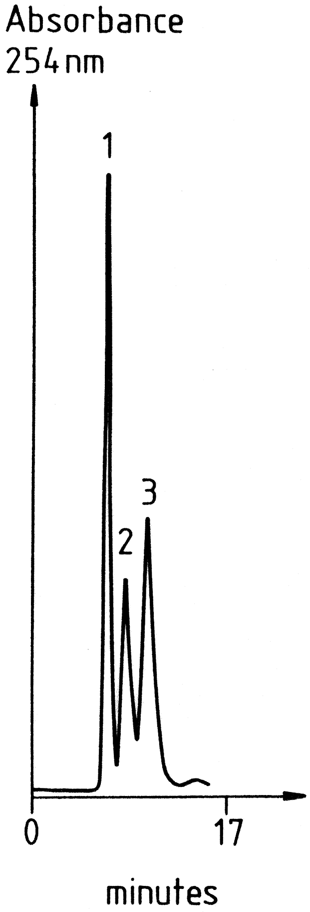


Fig. 2. Separation of 3-aminophenol (1), 2-aminophenol (2) and 4-aminophenol (3) on a zein-coated alumina column. Chromatographic conditions as in Fig. 1.

Table 2
Mean log k'_w values and relative standard deviations of ring-substituted phenol and aniline derivatives on silica and alumina supports covered by 2.5% zein[a]

Compound No.	Log k'_w			
	Al_{Zein}		Si_{Zein}	
	Mean	RSD (%)	Mean	RSD (%)
1	−0.65	0.12	0.35	0.27
2	−0.13	0.13	0.15	0.41
3	0.03	0.05	0.33	0.17
4	0.05	0.21	0.45	0.31
5	0.12	0.53	0.60	0.53
6	0.11	0.41	0.59	0.60
7	0.11	0.70	0.59	0.39
8	0.48	0.03	0.71	0.51
9	0.65	0.11	0.07	0.14
10	0.49	0.89	0.11	0.05
11	0.19	0.95	0.65	0.09
12	0.02	0.71	0.27	0.83
13	0.64	0.83	0.74	0.93
14	−0.18	0.70	0.01	0.19
15	−0.25	0.72	0.12	0.21
16	−0.20	0.82	0.03	0.32
17	−0.22	0.54	0.14	0.42
18	0.39	0.32	−0.03	0.35
19	0.95	0.45	1.90	0.07

[a] Numbers correspond to solutes in Table 1.

Table 3
Lipophilicity (R_{M0}) and specific hydrophobic surface area (b) of ring-substituted phenol and aniline derivatives determined on impregnated silica and alumina layers

Compound No.	Impregnation (%)							
	Silica				Alumina			
	2.5		10		2.5		10	
	R_{M0}	$b \cdot 10^2$	R_{M0}	$b \cdot 10^2$	R_{M0}	$b \cdot 10^2$	R_{M0}	$b \cdot 10^2$
1					0.16	1.83	0.67	1.86
2	−0.62	1.86	−0.35	1.56	0.65	1.83	0.67	1.86
3	−0.37	1.86	−0.09	1.63	−0.01	1.04	0.35	1.35
4	0.45	2.20	0.77	2.17	0.47	1.75	0.99	2.42
5	0.57	1.18	0.86	1.60	0.63	1.67	1.13	2.13
6	0.56	1.76	0.85	1.59	0.63	1.65	1.13	2.11
7	0.57	1.76	0.86	1.61	0.64	1.65	1.12	2.04
8	0.35	1.88	0.65	1.80				
9	1.03	1.77	1.30	1.16	0.81	0.85	1.32	1.37
10	0.83	1.51	1.04	0.89	0.64	0.74	1.13	1.24
11					0.20	0.38	0.69	0.94
12					0.01	2.43	0.28	1.51
13	−0.20	1.28	0.09	1.57	0.65	1.03	0.98	1.31
14	−0.34	1.59	−0.07	1.74	−0.20	1.09	0.16	1.63
15					0.38	1.86	0.48	1.97
16	0.22	1.87						
17	0.95	2.14	1.21	2.30	0.72	2.29	1.16	2.66
18	0.55	2.26	0.97	2.66	0.36	2.20	0.80	2.53
19	0.14	1.79	0.54	2.39	0.10	2.41	0.53	2.76

Table 4
Similarities and dissimilarities between the physicochemical parameters of ring-substituted phenol and aniline derivatives and their retention on zein-coated silica and alumina supports[a]

No. PC components	Eigen value	Variance explained (%)	Total variance explained (%)
1	6.04	30.20	30.20
2	4.27	21.37	51.57
3	3.03	15.15	66.72
4	2.16	10.82	77.54
5	1.45	7.24	84.78
6	1.12	5.64	90.41
7	0.95	4.76	95.17

[a] Results of principal component analysis. General parameters.

prepared by Dr. Barna Bordás (Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary).

3. Results and discussion

The separation of 3-aminophenol, 3-hydroxyphenol and 3-fluorophenol on zein-coated silica support and the separation of 3-aminophenol, 2-aminophenol and 4-aminophenol on zein-coated alumina support are shown in Figs. 1 and 2, respectively. The chromatograms show that the character and position of the substituents equally influence the retention of ring-substituted phenol derivatives on zein-coated supports that is zein selectively binds these compounds.

The mean log k'_w values of solutes and the RSDs

Table 5
Similarities and dissimilarities between the physicochemical parameters of ring-substituted phenol and aniline derivatives and their retention on zein-coated silica and alumina supports[a]

Parameter	No. of principal components						
	1	2	3	4	5	6	7
$R_{M0Al2.5}$	0.24	−0.63	−0.26	0.59	0.18	−0.26	0.08
$b_{Al2.5}$	0.68	0.25	−0.31	0.56	0.11	−0.09	−0.01
R_{M0Al10}	0.58	−0.61	0.03	0.42	0.28	0.04	0.08
b_{Al10}	0.84	0.20	−0.41	0.03	−0.05	0.19	0.03
$R_{M0Si2.5}$	0.80	0.09	0.37	−0.29	0.13	0.25	0.19
$b_{Si2.5}$	0.62	0.64	−0.24	−0.04	0.23	−0.09	−0.21
R_{M0Si10}	0.86	−0.11	0.37	−0.23	−0.11	0.07	0.07
b_{Si10}	0.43	0.75	−0.40	0.12	0.04	0.20	−0.02
Log k'_{Alzein}	−0.08	−0.12	0.49	0.46	−0.35	0.55	−0.21
Log k'_{Sizein}	−0.06	−0.48	0.69	0.35	0.20	0.19	0.25
π	0.84	0.29	0.28	−0.14	−0.18	−0.10	−0.11
H-Ac	−0.28	0.20	−0.47	−0.12	−0.13	0.14	0.72
H-Do	−0.89	0.25	−0.16	0.19	−0.04	−0.03	0.08
M-Re	−0.29	0.54	0.08	−0.23	0.46	0.46	0.09
F	−0.21	0.37	0.58	−0.31	0.15	−0.42	0.03
R	0.78	−0.36	−0.03	−0.23	0.14	−0.16	0.27
σ	−0.27	0.28	0.25	0.12	0.81	0.02	−0.07
Es	−0.10	0.75	−0.13	0.55	−0.03	−0.07	0.14
B_1	0.02	0.47	0.67	0.24	−0.26	−0.24	0.28
B_4	0.17	0.70	0.49	0.38	−0.09	−0.10	0.03

[a] Results of principal component analysis. Principal component loadings. For symbols see Experimental.

are compiled in Table 2. Considerable differences have been found among the log k values of solutes on zein-coated supports. Because the retention in water is related to the strength of the binding of solutes to zein, the differences indicate that the chemical structure of solutes markedly influences their interaction with the surface of zein molecules. Interestingly, the log k'_w values are different on zein-coated silica and alumina supports in the majority of cases a stronger binding to zein-coated silica has been observed. These results can be tentatively explained by the supposition that the adsorption character of the original support partially remains even after impregnation influencing the retention behavior of polar solutes liable to electrostatic interactions. The RSD values were lower than 1% in each instances proving the good reproducibility of the HPLC system and the good stability of the zein-coated supports.

The lipophilicity and specific hydrophobic surface area of solutes determined in the four TLC systems are compiled in Table 3. Blank sites in the Table indicate that the spot shape of the solutes was irregular in the given TLC system and the reliability of the measurement of R_F values was not high enough for the calculation of the relationship between retention and the composition of the mobile phase. It can be established from the data that the retention of solutes is higher on layers with higher amount of apolar coating on the surface. This phenomena indicates the involvement of hydrophobic interactive forces in the retention. Considerable differences can be observed between the hydrophobicity parameters of solutes determined on silica and alumina supports suggesting the marked influence of the adsorption centers of the original supports even after impregnation.

The results of PCA are summarized in Tables 4 and 5. Eigen values indicate the relative importance of the individual principal components. It is generally accepted that PCs having Eigen values over one have to be taken into consideration in the evaluation of the results of PCA. Variance explained represent the percent of variance explained by the individual PCs while total variance explained summarizes the variances explained by the individual PCs. Seven principal components explained the majority of variance, indicating that the information content of

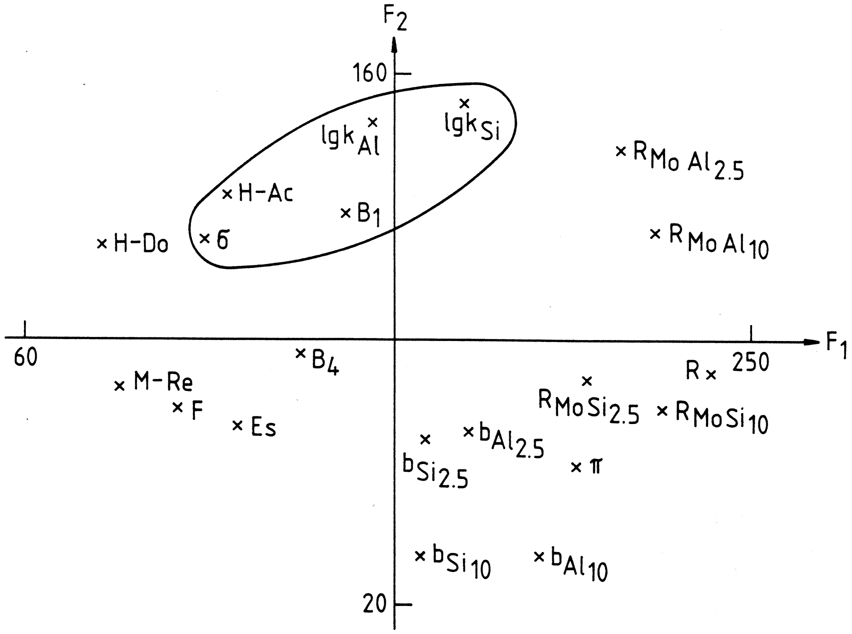


Fig. 3. Similarities and dissimilarities between the binding characteristics and measured and calculated physicochemical parameters of ring-substituted phenol and aniline derivatives. Two-dimensional non-linear map of PC loadings. Number of iterations: 143, maximum error: $7\cdot10^{-3}$. For symbols see Experimental.

the 20 original variables can be included into seven theoretical (background) variables with only 4.83% loss of information. Unfortunately, PCA does not prove the existence of such background variables as concrete physicochemical entities, only indicates their mathematical possibilities (Table 4). The loadings of log *k* values and the measured and calculated physicochemical parameters are widely distributed between the principal components suggesting that the binding of ring-substituted phenol and aniline derivatives to zein involves various interactive forces (Table 5).

The two-dimensional nonlinear map of principal component loadings is shown in Fig. 3. Axes F1 and F2 are dimensionless numbers without any concrete physicochemical meaning. They only define the site of the PC loadings and variables on a two-dimensional plane derived from a multidimensional map. The distribution of variables on the map entirely supports the previous conclusions. The log *k* values determined on zein-coated alumina and silica supports are near to each other, indicating the similarity (but not the identity) of the binding strengths observed on the supports. Steric and electrostatic parameters form a well-defined cluster with the log *k* values indicating again the involvement of steric correspondence and electrostatic forces in the interaction of solutes with zein. Measured and calculated hydrophobicity parameters are near to each other and far away from the cluster containing the log *k* values. The finding suggests that the various hydrophobicity parameters are similar to each other and hydrophobic interactive forces exert a negligible influence on the binding of polar solutes to zein.

4. Conclusion

It can be concluded from the data that the binding of ring-substituted phenol and aniline derivatives to the maize protein zein can be successfully determined by reversed-phase HPLC. The strength of protein–solute interaction mainly depends on the sterical correspondence between the solute and the substructures of the protein molecule accounting for the interaction and on the electrostatic interactive forces. Molecular hydrophobicity exerts a negligible influence on the strength of interaction.

Acknowledgements

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