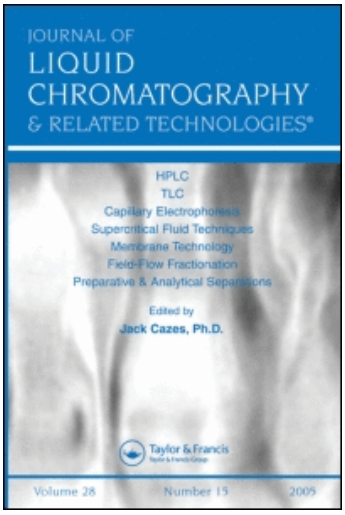


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# Effect of pH and Salts on the Binding of Ring-Substituted Phenol Derivatives to the Corn Protein Zein, Studied by Thin-Layer Chromatography

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## ABSTRACT

The interaction of seven ring-substituted phenol derivatives with the corn protein zein was studied by reversed-phase thin-layer chromatography (RP-TLC) carried out on zein-impregnated cellulose layers, and the effect of pH and salts on the strength and selectivity of the interaction was determined and elucidated by using spectral mapping techniques (SPM) and stepwise regression analysis (SRA). The binding of each phenol derivative to zein has been demonstrated. Calculations proved that the electron withdrawing capacity of substituents and the molecular hydrophobicity of phenol derivatives exert the highest influence on the phenol–zein binding indicating the mixed character of the interaction.

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*Key Words:* TLC; Zein-coated support; Phenol derivatives.

## INTRODUCTION

Because of their wide spread applications in various industrial processes[1] phenol and ring-substituted phenol derivatives are potential environmental pollutants occurring in wastewater,[1,2] environmental waters,[3] and soils.[4] Phenols can exert manyfold toxic effects,[5] they impair the viability of primary colonic epithelial cells,[6] are hematotoxic,[7] and influence enzyme production.[8] The noxious effect of phenols is mainly attributed to their binding to proteins, however, their interaction with DNA[9] has also been demonstrated. The association of phenols with bovine serum albumin[10] and arginase[11] has been recently established.

The character of the binding forces involved in the interaction of phenols with proteins has been vigorously investigated. Because of the high variety of proteins and phenols the results considerably depended on the protein–phenol pair under examination. It has been established that ionic bonding is predominant in the binding of phenolic compounds to canola proteins,[12] and hydrogen bonds play a decisive role in the binding of phenolic molecules to horseradish peroxidase.[13] The predominant importance of sterical[14] and electrostatical forces[15] in the protein–phenol interaction, has also been reported. However, the hydrophobic and nonspecific binding of alkyl- and alkoxy-phenols to human serum albumins has also been reported.[16]

Because of the high separation capacity and sensitivity, chromatographic methods have been frequently applied in the determination of molecular interactions.[17] These techniques have some advantages: they are rapid and the compounds to be studied need not be very pure, because their impurities separate under the chromatographic process. The use of thin-layer chromatography (TLC) for the study of interactions has marked advantages: the method is generally rapid, it is easy to carry out, makes possible the simultaneous determination of numerous interaction on one plate, and the amount of the more hydrophobic interactive compounds is extremely low.[18]

Spectral mapping technique (SPM), a multivariate mathematical–statistical method, has been developed for the separation of the strength and selectivity of biological activities.[19]

The method divides the information into two matrices using the logarithm of the original data. The first one is a vector containing the potency values related to the overall effect. The second matrix (selectivity map) contains the information concerning the spectra of activity (the qualitative characteristics of the effect).[20] Spectral mapping technique firstly calculates the logarithm of the members of the original data matrix, facilitating the evaluation of the final





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plots in terms of log ratios. Consecutively, SPM subtracts the corresponding column-mean and row-mean from each logarithmic element of the matrix, calculating potency values. The source of variation remaining in the centered data set can be evaluated graphically (selectivity map). Spectral mapping technique has been previously employed for the elucidation of the relationship between the chemical structure and fungicidal activity of nonionic surfactants,[21] for the study of the inhibitory effect of surfactants on sunflower downy mildew,[22] and for the characterization of stationary phases in HPLC.[23] As the selectivity matrices of SPM are generally multidimensional, they cannot be evaluated by visual methods. Nonlinear mapping technique (NLMAP) was developed for the reduction of the dimensionality of such matrices.[24]

## EXPERIMENTAL

The chemical names of ring-substituted phenol derivatives are compiled in Table 1. Microcrystalline cellulose for TLC and methanol of HPLC quality have been obtained from Merck (Darmstadt, Germany). Zein-coated cellulose stationary phase has been prepared by dissolving 1.0 g zein in the mixture of 160 mL of *n*-propanol and 40 mL of water at 70°C under continuous gentle stirring. After the dissolution of the protein, 20 g of microcrystalline cellulose was added and the mixture was stirred for 2 hours at the same temperature. Solvents have been removed at 70°C in vacuum. Plates of $20 \times 20$ cm containing 5 g of stationary phase have been prepared. Untreated cellulose plates served as controls. Phenol derivatives have been dissolved in acetone at the concentration of 5 mg $mL^{-1}$, and 2 μL of the solutions were spotted onto the plates. Phenol derivatives have been developed in distilled water as mobile

***Table 1.*** Chemical names of ring-substituted phenol derivatives.

| No. of phenol derivative | Chemical name |
|---|---|
| I | 4-Nitrophenol |
| II | 4-Aminophenol |
| III | 2-Aminophenol |
| IV | 3-Aminophenol |
| V | 3-Hydroxyphenol |
| VI | 4-Cyanophenol |
| VII | 2,6-Dimethoxyphenol |





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phase and in 0.16 M aqueous solutions of acetic acid, sodium acetate, sodium chloride, calcium chloride, and magnesium chloride, and in 0.05 M solution of NaCl. In order to assess the effect of organic modifier on the strength of phenol-zein binding developments have also been carried out in the mobile phase water : methanol (3 : 1, v/v). Unmodified cellulose stationary phase served as control in each instance. Plates have been developed in sandwich chambers (22 × 22 × 3 cm) at ambient temperature; the development distance was approximately 16 cm. After development, the plates have been dried at 105°C and the solutes have been detected by iodine vapors. Each experiment has been run in quadruplicate. The $R_M$ values characterizing the retention of solutes in reversed-phase thin-layer chromatography (RP-TLC) have been calculated by

$$R_M = \log\left(\frac{1}{R_f - 1}\right) \tag{1}$$

for each phenol derivative in each mobile phase. When the coefficient of variation of the parallel determinations was higher than 6% the $R_M$ value was omitted from the subsequent calculations. Higher $R_M$ values reflect stronger interaction between phenols and zein and between phenols and cellulose.

The strength and selectivity of the phenol–zein binding, and the strength and selectivity of the effect of mobile phase additives have been separated by SPM. Spectral mapping technique has been carried out twice:

1. The various mobile phases being the rows, and the phenol derivatives the columns. The potency values are related to the strength of the effect of mobile phase additives to the phenol–zein binding, the spectral map reflects the selectivity of the effect of mobile phase additives on the phenol–zein binding.
2. The various phenol derivatives being the rows, and the mobile phase additives the columns. The potency values are related to the strength of the phenol–zein binding, the spectral map reflects the selectivity of the binding of the individual phenols to zein.

In order to facilitate the visual evaluation of the multidimensional spectral maps their dimensionality has been reduced to two by NLMAP. Iterations were carried out to the point where the differences between the last two iterations was less than $10^{-8}$.

The effect of various molecular parameters of mobile phase additives and that of the interacting phenol derivatives on the strength and selectivity of interaction was calculated by stepwise regression analysis (SRA). Calculations were performed six times.





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Stepwise regression analysis 1–3: the potency values, the first and second coordinates of the two-dimensional nonlinear spectral map of mobile phase additives were individually the dependent variables. The concentration of the individual mobile phase additives, the total ion concentration, cation charge, and cation redox potential were the independent variables in each instance.

Stepwise regression analysis 4–6: the potency values, the first and second coordinates of the two-dimensional nonlinear spectral map of phenol derivatives were individually the dependent variables. The independent variables were, in each instance, the following calculated physicochemical parameters of phenol derivatives: $\pi$ = Hansch-Fujita's substituent constants characterizing hydrophobicity, H-Ac and H-Do = indicator variables for proton acceptor and proton donor properties, respectively, M-RE = molar refractivity, $F$ and $R$ = Swain and Luton's electronic parameters characterizing the inductive and resonance effects, $\sigma$ = Hammett's constant characterizing the electron-withdrawing power of the substituent, $E_s$ = Taft's constant characterizing the steric effects of substituents, and $B_1$ and $B_4$ = Sterimol width parameters determined by distance of substituents at their maximum point perpendicular to attachment. The parameters of solutes were calculated according to the additivity rule from the fragmental constants.

The number of accepted independent variables was not limited and the acceptance limit was set to 95% significance level in each instance.

Software for SRA named DRUGIDEA was purchased from Compudrug Ltd (Budapest, Hungary), softwares for SPM and NLMAP were facilitated by Dr. Barna Bordás, Plant Protection Institute, Hungarian Academy of Sciences (Budapest, Hungary).



## RESULTS AND DISCUSSION

The $R_M$ of ring substituted phenol derivatives determined in various chromatographic systems are compiled in Table 2. The data in Table 2 demonstrate that the $R_M$ values show marked variations according to the chemical structure of solute molecule, the type of salt and pH of the mobile phase, and the character of the stationary phase (impregnated or unimpregnated). The deviations between the $R_M$ values determined on unimpregnated and zein-impregnated cellulose stationary phases prove that zein really influences the retention of phenol derivatives by interacting with them. The considerable differences between the effects of salts and pH suggest that not only the pH and the concentration of salts but also the type of cations, exerts a remarkable influence on the strength of interaction of phenols with zein.

The potency values (related to the strength of zein–phenol interaction) are compiled in Table 3. The results entirely support our previous qualitative conclusions. The high differences between the potency values of phenols



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***Table 2.*** $R_M$ values of ring-substituted phenol derivatives in various mobile phases.

| No. | Stationary phase | Mobile phase additive | No. of phenol derivatives | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | I | II | III | IV | V | VI | VII |
| 1 | Z | 0 | 0.30 | −0.07 | −0.21 | −0.26 | −0.14 | 0.03 | −0.23 |
| 2 | C | | −0.28 | −0.25 | −0.36 | −0.36 | −0.38 | −0.49 | −0.50 |
| 3 | Z | Methanol | −0.04 | −0.16 | −0.31 | −0.46 | −0.50 | −0.34 | −0.57 |
| 4 | C | | −0.49 | −0.46 | −0.47 | −0.54 | −0.65 | −0.66 | −0.71 |
| 5 | Z | 0.05 M | 0.06 | −0.69 | −0.66 | −0.58 | −0.35 | −0.13 | −0.30 |
| 6 | C | NaCl | −0.10 | −0.32 | −0.41 | −0.39 | −0.37 | −0.28 | −0.37 |
| 7 | Z | 0.16 M | 0.08 | −0.73 | −0.78 | −0.78 | −0.46 | −0.26 | −0.53 |
| 8 | C | NaCl | −0.14 | −0.26 | −0.48 | −0.50 | −0.38 | −0.34 | −0.38 |
| 9 | Z | 0.16 M | 0.27 | −0.61 | −0.66 | −0.65 | −0.22 | −0.06 | −0.26 |
| 10 | C | Acetic acid | −0.13 | −0.40 | −0.54 | −0.53 | −0.44 | −0.34 | −0.47 |
| 11 | Z | 0.16 M | 0.31 | −0.33 | −0.25 | −0.27 | −0.15 | 0.07 | −0.16 |
| 12 | C | Sodium acetate | −0.49 | −0.64 | −0.64 | −0.57 | −0.57 | −0.45 | −0.51 |
| 13 | Z | 0.16 M | 0.24 | −0.69 | −0.61 | −0.62 | −0.38 | −0.18 | −0.36 |
| 14 | C | $MgCl_2$ | −0.06 | −0.36 | −0.47 | −0.47 | −0.30 | −0.33 | −0.36 |
| 15 | Z | 0.16 M | −0.23 | −0.64 | −0.53 | −0.56 | −0.28 | −0.26 | −0.09 |
| 16 | C | KCl | −0.11 | −0.39 | −0.59 | −0.44 | −0.35 | −0.33 | −0.35 |
| 17 | Z | 0.16 M | 0.14 | −0.75 | 0.58 | −0.67 | −0.31 | −0.10 | −0.38 |
| 18 | C | $CaCl_2$ | −0.11 | −0.43 | −0.51 | −0.43 | −0.34 | −0.27 | −0.32 |

*Key:* Z, zein-coated cellulose; C, unimpregnated cellulose.
*Note:* Roman numbers refer to phenol derivatives in Table 1.

***Table 3.*** Effect of pH and salts on the relative strengths of phenol–zein and phenol–cellulose interactions.

| Ring-substituted phenol derivatives | | Chromatographic systems | | Chromatographic systems | |
|---|---|---|---|---|---|
| No. | Potency | No. | Potency | No. | Potency |
| I | −0.18 | 1 | −0.21 | 10 | −1.08 |
| II | −1.93 | 2 | −0.99 | 11 | −0.29 |
| III | −1.86 | 3 | −0.90 | 12 | −1.49 |
| IV | −2.14 | 4 | −1.50 | 13 | −0.98 |
| V | −1.55 | 5 | −1.00 | 14 | −0.98 |
| VI | −1.11 | 6 | −0.85 | 15 | −0.97 |
| VII | −1.61 | 7 | −1.31 | 16 | −0.97 |
| | | 8 | −0.94 | 17 | −0.56 |
| | | 9 | −0.83 | 18 | −0.91 |

*Note:* Potency values (arbitrary units) calculated by the spectral mapping technique. Roman and arabic numbers refer to phenol derivatives in Table 1 and chromatographic systems in Table 2, respectively.



illustrate again the marked role of molecular structure on the interaction of phenols with zein. The deviations among the potency values of chromatographic systems further demonstrate the impact of zein coating, pH, and the presence of salts on the strength of interaction.

The two-dimensional nonlinear selectivity map of chromatographic systems is shown in Fig. 1. The scales of the map are dimensionless numbers, indicating only the distribution of points on the two-dimensional plane. Cellulose stationary phases (even numbers) form a well-defined cluster, suggesting that pH and salts exert a similar effect on the selectivity of binding of phenol derivatives to cellulose. Opposite to cellulose, the points representing the zein-coated stationary phase are widely distributed on the map, indicating that the mobile phase additives influence differently the selectivity of binding of phenol derivatives to zein. Interestingly, the effect of pH (acidic: point 9; alkaline: point 11) is similar to that of the majority of salts. Mobile phases containing salts are widely distributed on the map, suggesting again that salts exert a different influence on the selectivity of phenol–zein interaction, and not only the concentration but also the physicochemical parameters of cations may have a marked impact on the selectivity of interaction.

Phenol derivatives did not form clusters according to the character of the individual substituents on the two-dimensional non-linear selectivity map



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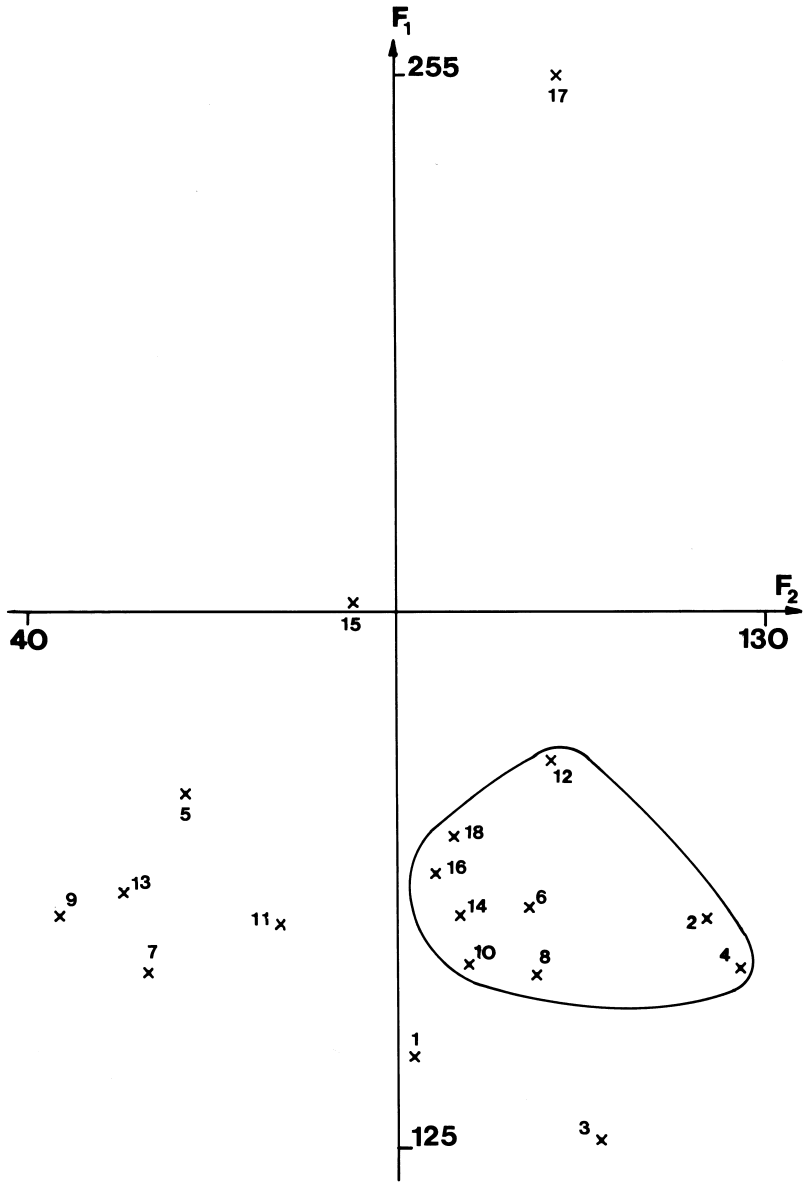


***Figure 1.*** Selectivity of the effect of pH and salts on the phenol–zein and phenol–cellulose interaction. Two-dimensional non-linear selectivity map. No of iterations: 119; Maximum error: $1.05 \times 10^{-2}$. Numbers refer to chromatographic systems in Table 2.





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(data not shown), illustrating that the impact of each substituent on the selectivity of zein–phenol interaction is similar, and the distribution observed is the interplay of the contributions of each substituent to the selectivity.

The parameters of significant relationships between the binding parameters of phenol–zein interaction and the physico-chemical parameters of phenols and mobile phase additives are compiled in Table 4. In the majority of cases, SRA found significant relationships between the dependent and independent variables, the significance level being always over 95% (compare calculated and tabulated $F$ values). The independent variables explain 20–85 % of the total variance (see $r^2$ values). The low ratio of variance explained by equations I, II, and III may be due to the fact that other physicochemical parameters of mobile phase additives not included in the calculations may also have a significant influence on the binding parameters. The concentration of



***Table 4.*** Parameters of linear relationships between the physico-chemical parameters of mobile phase additives and their effect on the binding of phenols and the physico-chemical parameters of phenols on their binding to zein and cellulose.

Mobile phase additives ($n = 18$)

I Potency $= a + b_1$ Methanol (vol.%) $+ b_2$ Redox potential
II Spm1 $= a + b_1$ Concentration of $CaCl_2$
III Spm2 $= a + b_1$ Redox potential

Phenol derivatives ($n = 7$)

IV Potency $= a + b_1 \pi$
V Spm1 $= a + b_1 \sigma$
VI Spm2 = not significant

| | No. of equation | | | | |
|---|---|---|---|---|---|
| Parameters | I | II | III | IV | V |
| $a$ | −0.30 | 153.5 | 108.7 | −0.07 | 146.5 |
| $b_1$ | $-3.59 \times 10^{-2}$ | 370.6 | 9.79 | 1.56 | 74.64 |
| $s_{b1}$ | $1.16 \times 10^{-2}$ | 107.4 | 4.52 | 0.29 | 21.89 |
| $b_2$ | 0.24 | — | — | — | — |
| $s_{b2}$ | 0.08 | — | — | — | — |
| $b_1$ (%) | 51.70 | — | — | — | — |
| $b_2$ (%) | 48.30 | — | — | — | — |
| $r^2$ | 0.4230 | 0.4265 | 0.2266 | 0.8510 | 0.6994 |
| $F_{\text{calc.}}$ | 5.50 | 11.90 | 4.69 | 28.55 | 11.63 |
| $F_{95\%}$ | 3.68 | 4.49 | 4.49 | 6.61 | 6.61 |

*Note:* Results of SRA analysis. Spm1 and Spm2, first and second co-ordinates of the two-dimensional nonlinear selectivity maps.



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methanol decreased the strength of interaction, while it increased with increasing redox potential of cations. The normalized slope values ($b_i$%) indicated that the impact of these two independent variables on the strength of interaction is commensurable. The results suggest that the molecular lipophilicity of phenol derivatives influences the strength of zein–phenol interaction, and the selectivity depends on the electron-withdrawing power of substituents. The fact that the both hydrophobic and hydrophilic parameters exert a marked impact on the phenol–zein interaction indicates the involvement of more than one type of interactive forces in the binding. It can be assumed that the apolar ring structure of solutes can bind to the hydrophobic side chain of amino acids (van der Waals or stacking interactions), and the polar head groups can interact with the hydrophilic peptide bonds or with the polar side chains of basic or acidic amino acids.

It can be concluded from the data, that the binding of ring-substituted phenol derivatives to the corn protein zein can be successfully studied by RP-TLC carried out on zein-coated plates. The strength of interaction depends on both the electrostatic parameters and hydrophobicity of the solutes. Concentration and character of salts and pH considerably influence the strength and selectivity of such interactions.



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