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## COMBINED EFFECTS OF pH AND SURFACE-ACTIVE-ION CONCENTRATION IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

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### SUMMARY

The combined effects of pH (3.6–6.0) and octylamine hydrochloride concentration (0–5.0 mM) in methanol–water (20:80) eluents were determined for hydrocinnamic, *trans*-cinnamic, phenylacetic, *trans-p*-coumaric, *trans*-ferulic, *trans*-caffeic and vanillic acids; for phenylethylamine; and for phenylalanine. The effects are described by a simple ion-interaction model that does not require ion-pair formation in either phase and is not based upon classical ion exchange. The simplicity and generality of the mathematical forms of the model make it useful for predicting retention behavior.

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### INTRODUCTION

The tremendous success of reversed-phase high-performance liquid chromatography (HPLC) employing hydrocarbonaceous bonded stationary phases is largely attributable to the rich variety of factors that can be adjusted in the polar mobile phase, *e.g.*, pH, ionic strength, polarity, dielectric constant, hydrogen bonding capability, and concentration of surface active ions<sup>1</sup>. Although research is still being directed toward improvements in bonded phases<sup>2,3</sup>, most analysts do not have the necessary laboratory facilities to make direct chemical modifications of the stationary phase; as a practical matter, they are able to adjust only the composition of the eluent to improve their separations<sup>4</sup>. Thus, much current research is appropriately directed toward a fundamental understanding of how changes in eluent composition affect the chromatographic separation process.

The pH<sup>5-9</sup> and the concentration of surface-active ions<sup>10-26</sup> in the eluent are two factors that have each been shown to be especially useful for regulating the retention times of weak acids and weak bases. This paper presents the results of an experimental study designed to determine the *combined* effects of pH and surface-active ion concentration on the reversed-phase liquid chromatographic behavior of weak acids, weak bases and zwitterionic compounds.

## THEORETICAL

*Hydrogen ion dependence*

In the absence of an intentionally added surface-active ion, the observed retention time ( $t_R$ ) of a weak acid or a weak base as a function of hydrogen ion concentration can be described by the relationship

$$t_R = f_{HA}t_{HA} + f_A t_A \quad (1)$$

In eqn. 1,  $t_{HA}$  is the retention time of the solute when it is completely in the protonated form,  $t_A$  is its retention time when it is completely in the unprotonated form, and  $f_{HA}$  and  $f_A$  are the fractions of compound in the protonated and unprotonated forms, respectively, for any intermediate condition<sup>4-8</sup>

$$f_{HA} = \frac{[H^+]}{[H^+] + K_a} \quad (2)$$

$$f_A = \frac{K_a}{[H^+] + K_a} \quad (3)$$

where  $K_a$  is the acid dissociation constant for the protonated form of the solute HA.

*Surface-active ion dependence*

Bidlingmeyer *et al.*<sup>25</sup> have suggested that neither the "ion-pair" model<sup>11,14,19,22</sup> nor the "ion-exchange" model<sup>17,20,23,24</sup> adequately describes chromatographic phenomena observed in reversed-phase HPLC systems containing surface-active ions intentionally added to the mobile phase. Rather, they have proposed an ion-interaction mechanism<sup>25</sup> which does not require classical ion-exchange "sites" or ion-pair formation in either phase. Important features of the ion-interaction model are that (a) adsorbed surface active ions (ion-interaction reagent, IIR) are responsible for a charged primary ion layer at the surface of the stationary phase; (b) the charged primary ion layer electrostatically attracts or repels solute ions of opposite or similar charge, respectively; (c) the charged primary ion layer does not exert an effect upon uncharged molecules; and (d) other differences in distribution behavior can be explained by forces that are eluophilic (having an affinity for the mobile phase), eluophobic (having an aversion for the mobile phase), adsorbophilic (having an affinity for the bonded stationary phase), and adsorbophobic.

According to the ion-interaction model, the increased (or decreased) retention of a solute ion is proportional to the amount of charge in the primary ion layer; this, in turn, is proportional to the amount of adsorbed IIR; and the amount of adsorbed IIR is related to the concentration of IIR in the eluent by an adsorption isotherm such as the Freundlich isotherm<sup>26,30</sup>. Thus, the effect of IIR on the retention of a solute ion can be described by eqn. 4

$$t_R = t_c + \beta[IIR]^{1/n} \quad (4)$$

where  $t_c$  is the retention time of the charged solute ion in the absence of intentionally added IIR, and  $\beta$  and  $n$  are parameters of the Freundlich isotherm. If the IIR and the solute ion are of opposite charge, then  $\beta$  will be positive indicating an increased

retention of the solute ion caused by electrostatic attraction; if the IIR and the solute ion are of similar charge, then  $\beta$  will be negative indicating a decreased retention of the solute ion caused by electrostatic repulsion.

If an intentionally added surface-active ion is a weak base (e.g., octylamine hydrochloride), then the fractions of surface-active compound existing in the protonated form ( $f_{HS}$ ) and unprotonated form ( $f_S$ ) are given by

$$f_{HS} = \frac{[H^+]}{[H^+] + K_s} \quad (5)$$

$$f_S = \frac{K_s}{[H^+] + K_s} \quad (6)$$

where  $K_s$  is the acid dissociation constant of the protonated form of the surface-active ion HS. Because it is the charged form of the IIR (HS in this case) that interacts with the charged solute, eqn. 4 may be rewritten as

$$t_R = t_c + f_{HS}\beta[IIR]^{1/n} \quad (7)$$

where [IIR] represents the analytical concentration of the weak base ion-interaction reagent.

#### Combined effects of pH and IIR

In this paper, the ion-interaction mechanism is extended to include effects caused by variations in the hydrogen ion concentration. For weak acid solutes that are uncharged in their protonated form (e.g., hydrocinnamic acid) and for ion-interaction reagents that are positively charged in their protonated form (e.g., octylamine)

$$t_R = f_{HA}t_{HA} + f_A(t_A + f_{HS}\beta[IIR]^{1/n}) \quad (8)$$

or

$$t_R = f_{HA}t_{HA} + f_A t_A + f_A f_{HS}\beta[IIR]^{1/n} \quad (9)$$

That is, the fraction of weak acid that exists in the uncharged protonated form ( $f_{HA}$ ) makes a contribution ( $t_{HA}$ ) to the observed retention time that is independent of the concentration of IIR. However, the fraction of weak acid that exists in the negatively charged unprotonated form ( $f_A$ ) contributes to the observed retention time in two ways: a contribution ( $t_A$ ) that characterizes its retention time in the absence of ion-interaction reagent ( $t_c$  in eqn. 7), and a contribution ( $f_{HS}\beta[IIR]^{1/n}$ ) that characterizes the proportionally increased retention time caused by the IIR that is present in its positively charged protonated form.

By analogous reasoning, for weak base solutes (e.g., phenylethylamine) and weak base ion-interaction reagents (e.g., octylamine) that are both positively charged in their protonated forms,

$$t_R = f_{HA}t_{HA} + f_{HA}f_{HS}\beta[IIR]^{1/n} + f_A t_A \quad (10)$$

## EXPERIMENTAL

### *Chromatographic system*

The chromatographic system consisted of a Model 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.), 5 cm  $\times$  4 mm I.D. Bondapak  $C_{18}$ /Parasil pre-column (Waters), 30 cm  $\times$  4 mm I.D.  $\mu$ Bondapak  $C_{18}$  main column (Waters), and a Model SP8200 ultraviolet (UV) detector (Spectra-Physics, Santa Clara, CA, U.S.A.) operated at 254 nm. A Model 70-10 automatic sample injection valve equipped with a Model 70-01 pneumatic activator (Rheodyne, Berkeley, CA, U.S.A.) was used to inject 20- $\mu$ l volumes of samples. Precolumn and column temperatures were held at  $25.0 \pm 0.1^\circ\text{C}$  by a Model FK constant temperature circulating bath (Haake, Saddle Brook, NJ, U.S.A.). Mobile phase flow-rate was maintained at  $2.02 \pm 0.02$  ml  $\text{min}^{-1}$ . The time equivalent of the void volume ( $t_0 = 1.783$  min) was determined by injecting 20  $\mu$ l of water and measuring the time from injection to the first deviation from baseline.

### *Additional instrumentation*

The analog UV detector output was recorded by a Model 281 strip-chart recorder (Soltex, Encino, CA, U.S.A.). Simultaneously, the signal from the detector was digitized by a Model ADC-12QZ analog-to-digital converter (Analog Devices, Norwood, MA, U.S.A.) interfaced to a Model 9830A digital computer (Hewlett-Packard, Calculator Products Division, Loveland, CO, U.S.A.). Chromatograms less than 60 min long were digitized at 1-sec intervals, longer chromatograms were digitized at 2-sec intervals. Chromatograms were drawn from digitized data on a Model 9862A plotter (Hewlett-Packard).

### *Experimental design*

A four-level, two-factor ( $4^2$ ) factorial design<sup>27,28</sup> was used to specify eluent compositions of 16 different mobile phases corresponding to all combinations of four pH values (3.6, 4.4, 5.2 and 6.0) and four concentrations of surface-active ion (0, 1.5, 3.0 and 5.0 mM). The experimental order of evaluating the eluents was randomized to minimize the confounding of time trends with factor effects<sup>29</sup>. Data are not included for the combination pH 4.4 and 3.0 mM surface-active-ion concentration because of discrepant results<sup>27</sup>.

### *Mobile phases and samples*

Mobile phases were prepared by adding octylamine hydrochloride (Aldrich, Milwaukee, WI, U.S.A.) to 200 ml of HPLC grade methanol (Fisher Scientific, Chemical Manufacturing Division, Fair Lawn, NJ, U.S.A.) in a 1-l volumetric flask. A 10-ml volume of 1 M acetic acid (Fisher) and 780 ml of distilled water were added; 1 M  $\text{HClO}_4$  (Fisher) or 1 M NaOH (Fisher) was added dropwise until a glass electrode indicated the desired pH. The volumetric flask was then brought to volume with distilled water.

Solutions containing individual solutes (Fisher; or Sigma, St. Louis, MO, U.S.A.) were prepared in methanol-water (20:80) at a concentration of approximately 0.2 mg  $\text{ml}^{-1}$ .

Mobile phases and sample solutions were aspirated through 0.47- $\mu\text{m}$  cellulose acetate filters (HAW04700; Millipore, Bedford, MA, U.S.A.) and degassed in a Model ME4.6 ultrasonic bath (Mettler Electronics, Anaheim, CA, U.S.A.) before use.

## RESULTS AND DISCUSSION

Table I contains observed retention times for each of the nine solutes at each of the 15 combinations of pH and [IIR]. Table I also contains the best non-linear least squares estimates of the parameters of corresponding mathematical models<sup>31</sup>. The standard deviation of residuals ( $s$ ) divided by the average retention time ( $\bar{t}_R$ ) is a relative measure of the goodness of fit of the mathematical model to the experimental data. Fig. 1 plots the experimental data and the fitted model (eqn. 9) for a representative solute, hydrocinnamic acid, and demonstrates the good agreement between the fitted model and the experimental data. Fig. 2 shows pseudo-three-dimensional plots<sup>32</sup> of estimated retention time as a function of both pH and [IIR] for all nine solutes.

*Weak acids*

In the absence of ion-interaction reagent (along the near left edge of the surfaces in Fig. 2), each of the seven weak acids studied (hydrocinnamic, *trans*-cinnamic, phenylacetic, *trans-p*-coumaric, *trans*-ferulic, *trans*-caffeic, and vanillic acids) shows the usual effect of pH. At low pH, each acid exists almost completely in the protonated, uncharged form and has a relatively high affinity for the stationary phase and a relatively low affinity for the mobile phase; this causes the acid to move slowly through the reversed-phase column. At high pH, each acid exists almost completely in the unprotonated, negatively charged form; the carboxylate group (and thus the entire molecule) is less adsorbophilic and more eluophilic which causes the charged molecule to move more rapidly through the column. At intermediate pH values, the retention time reflects the relative fractions of conjugate acid and conjugate base forms and the  $t_R$  vs. pH relationship exhibits the usual sigmoidal behavior<sup>5,6</sup>.

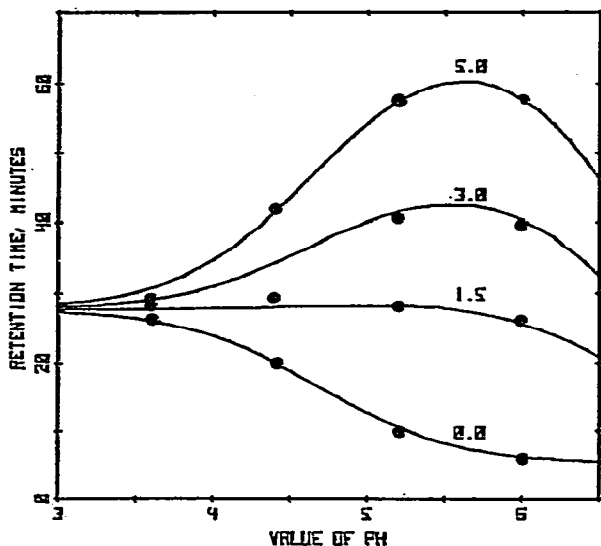


Fig. 1. Effect of pH on retention time of hydrocinnamic acid at different levels of surface-active ion (octylamine hydrochloride) concentration.

TABLE I  
RETENTION TIMES AND PARAMETER ESTIMATES FOR SOLUTES

<i>pH</i>	[IIR] (mM)	Observed retention time (min)									
		Hydro- cinnamic- acid	trans- Cinnamic acid	Phenyl- acetic acid	trans- <i>p</i> - Coumaric- acid	trans- Ferulic acid	trans- Caffeic acid	Vanillic acid	Phenyl- ethyl- amine	Phenyl- alanine	
3.6	0.0	26.05	42.39	10.92	15.53	20.92	9.17	7.65	5.48	2.94	
	1.5	28.53	47.67	13.43	15.78	21.23	9.84	7.98	1.67	2.74	
	3.0	29.12	49.10	14.31	15.31	20.51	9.51	7.78	1.66	2.76	
	5.0	29.05	50.14	14.73	15.08	20.24	9.93	7.69	1.65	2.79	
4.4	0.0	20.27	27.50	7.44	11.61	15.20	7.19	5.62	4.84	2.81	
	1.5	29.62	44.20	14.48	13.82	18.25	8.82	7.05	1.88	2.78	
	3.0	—	—	—	—	—	—	—	—	—	
	5.0	41.91	67.15	23.07	16.41	21.96	12.05	8.42	1.65	2.82	
5.2	0.0	10.16	11.24	4.07	5.57	6.90	3.87	3.07	5.07	2.79	
	1.5	28.05	36.37	13.32	9.68	12.64	6.71	5.11	2.22	2.81	
	3.0	40.62	54.98	19.38	12.69	16.71	9.07	6.51	1.96	2.82	
	5.0	57.70	78.53	27.35	16.15	21.41	12.53	8.10	1.80	2.84	
6.0	0.0	6.40	6.72	2.98	3.53	4.11	2.64	2.25	6.17	2.80	
	1.5	26.27	32.30	12.52	8.07	10.48	5.93	4.41	2.43	2.84	
	3.0	39.64	50.69	18.31	11.20	14.69	9.64	5.83	2.12	2.85	
	5.0	57.98	73.97	26.08	15.13	20.39	12.53	7.57	1.93	2.88	
<i>pK<sub>a</sub></i>		4.69	4.45	4.29	4.72	4.71	4.60	4.62	7.63	2.22	
<i>t<sub>HR</sub></i>		27.78	47.86	12.85	15.95	21.48	9.99	8.08	5.24	4.96	
<i>t<sub>A</sub></i>		5.23	5.19	2.96	2.61	2.88	2.36	1.89	26.97	2.82	
<i>β</i>		17.09	22.17	8.09	3.95	5.36	2.63	1.97	-3.27	-2.57	
<i>pK<sub>s</sub></i>		6.78	6.77	6.78	6.98	7.11	7.31	6.86	—	—	
<i>n</i>		1.23	1.24	1.33	1.29	1.29	1.12	1.36	14.02	3.21	
<i>s</i>		0.940	1.33	0.624	0.464	0.648	0.429	0.228	0.267	0.040	
<i>f<sub>r</sub></i>		31.42	44.86	14.83	12.37	16.38	8.63	6.36	2.84	2.82	
<i>s/f<sub>r</sub></i>		0.030	0.030	0.042	0.038	0.040	0.050	0.036	0.094	0.014	

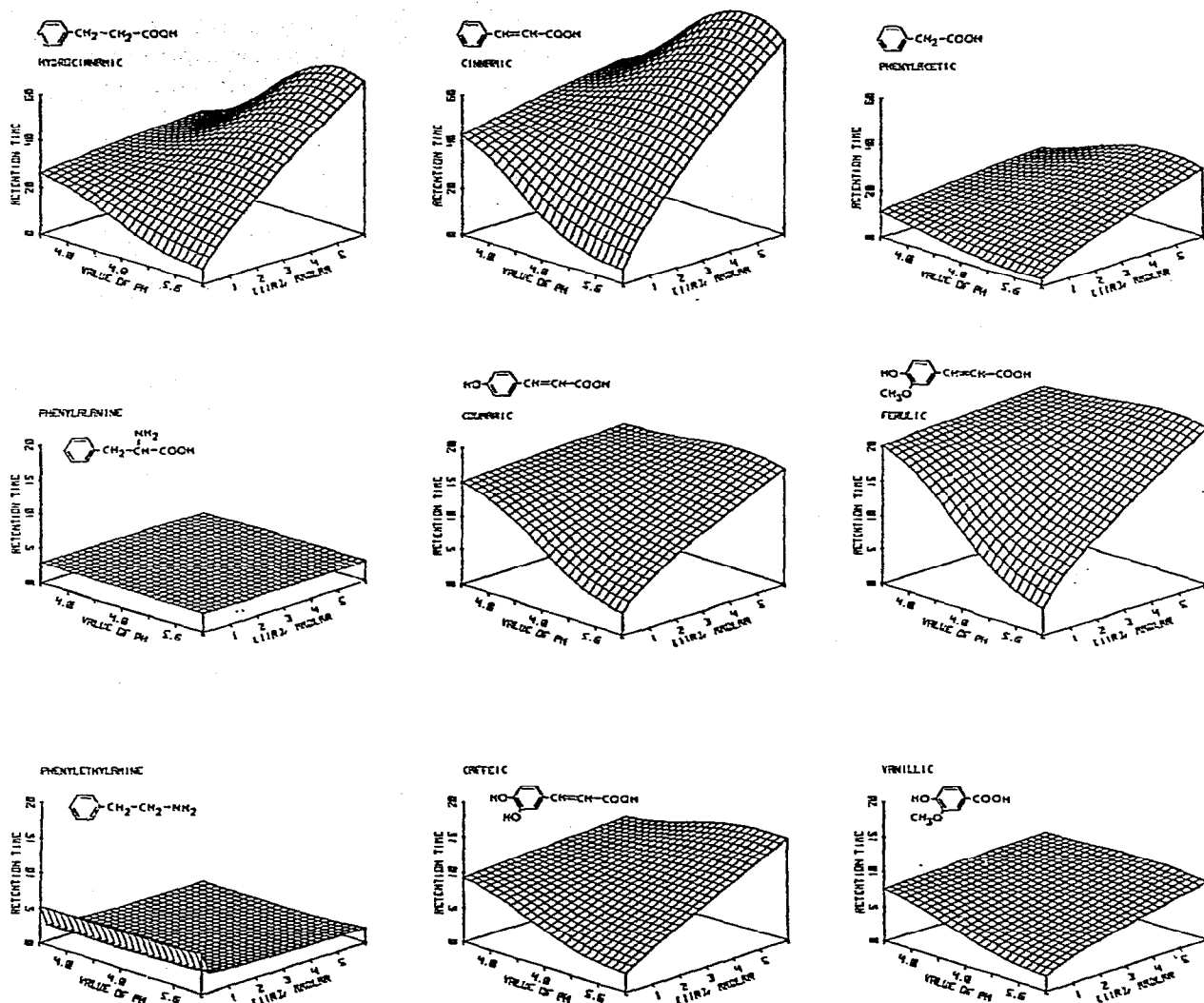


Fig. 2. Combined effects of pH and surface-active ion concentration (IIR) on the reversed-phase liquid chromatographic behavior of weak acids, weak bases, and zwitterionic compounds. See text for discussion.

At low pH (along the far left edge of the surfaces in Fig. 2), the retention time of each of the seven weak acids is essentially independent of the concentration of ion-interaction reagent in the eluent. This supports the findings of Bidlingmeyer *et al.*<sup>25</sup> that the retention of uncharged molecules is not influenced by the presence of surface-active ions. Schill<sup>33</sup> has recently shown a similar result for acetylsalicylic acid at pH 3.0.

However, at high pH (along the near right edge of the surfaces in Fig. 2), the retention of each of the seven weak acids is clearly affected by the concentration of IIR; the relationship between  $t_R$  and [IIR] is described by a Freundlich-type equation

(eqn. 7). Schill<sup>33</sup> has shown similar behavior for acetylsalicylic acid at pH 6.0. Presumably, the negatively charged conjugate base form of the solute now experiences an additional electrostatic interaction with the positively charged primary ion layer created by the adsorbed surface-active ions<sup>25</sup>.

In the presence of relatively large concentrations of IIR ( $\approx 5$  mM; along the far right edge of the surfaces in Fig. 2), each of the seven weak acids shows an effect caused by pH that is essentially opposite to that exhibited in the absence of IIR. As the pH is increased, a greater fraction of the acid exists in the unprotonated, negatively charged conjugate base form which strongly interacts with the positively charged adsorbed primary ion layer. This results in a net increase in retention time as the pH is increased.

Inspection of the response surfaces in Fig. 2 shows that at high concentrations of IIR, the retention time of each of the seven weak acids begins to decrease again near the highest pH values shown (see also Fig. 1). A possible explanation for this "foldover" is that the concentration of hydrogen ions in the eluent is so low that the adsorbed ion-interaction reagent is becoming appreciably uncharged and thus contributes a lesser amount of positive charge to the primary ion layer. Although the mechanistic model (eqn. 9) does take this into account and does adequately fit the observed experimental data, estimated values of  $pK_s$  are  $7.0 \pm 0.3$  (see Table I), approximately three log units more acidic than the value of 10.65 normally associated with octylamine<sup>34</sup>. It is not clear why the estimated values of  $pK_s$  are so low, but a different chemical mechanism of the same mathematical form might be involved (e.g., deprotonation of exposed  $-\text{Si}-\text{OH}$  groups on the surface of the stationary phase).

#### Weak base

The  $pK_a$  of phenylethylamine, a weak base, is 9.84 (ref. 35); over the pH range used in this study, phenylethylamine is expected to exist almost completely in its protonated, positively charged conjugate acid form (phenylethylammonium ion). Thus, in the absence of surface-active ions (see the near left side of the surface in Fig. 2), the retention of positively charged phenylethylammonium ion is relatively short and is relatively insensitive to changes in pH.

In the presence of positively charged surface-active octylammonium ions, the positively charged phenylethylammonium solute ion is repelled by the octylammonium ions on the surface of the stationary phase and the solute ion is eluted from the column very rapidly. Under some conditions, phenylethylamine elutes in less than the solvent void volume (see Table I); this effect has been observed previously<sup>4,15,25,36,37</sup>, and is attributed to a charge-exclusion phenomenon that makes some of the pore and interstitial volumes inaccessible to the charged solute.

A large covariance between  $K_a$  and  $K_s$  in the fitted model (eqns. 2, 3, 5 and 10) prevented convergence. However, removing  $K_s$  by setting  $f_{\text{HS}} = 1$  in eqn. 10 allowed convergence. Parameters of the fitted model (see Table I) suggest that the  $pK_a$  of phenylethylammonium ion (or, alternatively, of octylammonium ion) is 7.63, several log units more acidic than the accepted literature values<sup>34,35</sup>. Again, it is not clear why the estimated  $pK_a$  is so low; the observed effect is probably caused by a different chemical mechanism. The sign of the estimated value of  $\beta$  ( $-3.27$ ) is negative, as expected. In this case, the exceptionally high value of  $n$  (14.02) is



probably more a measure of the effectiveness of electrostatic repulsion than a measure of the adsorption of IIR.

### Zwitterion

The  $pK_a$  values of phenylalanine, a difunctional compound, are 2.16 and 9.15 (ref. 38); over the pH range used in this study, phenylalanine is expected to exist almost completely in its zwitterionic form—an ion possessing one positive charge ( $-\text{NH}_3^+$ ) and one negative charge ( $-\text{COO}^-$ ), yet having a net overall charge of zero. As seen in Table I and Fig. 2, the retention of phenylalanine is relatively unaffected by the presence of surface-active ions. Apparently the attractive and repulsive electrostatic forces that might exist between the positively charged primary ion layer and the  $-\text{COO}^-$  and  $-\text{NH}_3^+$  groups are cancelled. Thus, the zwitterionic phenylalanine behaves as an uncharged molecule<sup>25</sup>.

Because the zwitterion is not affected by the IIR, the effect of deprotonated octylammonium is not observed, and  $f_{HS}$  is set equal to unity in eqn. 10. The parameters of the fitted model are given in Table I. The estimated  $pK_a$  (2.22) is close to the accepted literature value of 2.16 (ref. 38). The values of  $\beta$  and  $n$  are estimated with large imprecisions and should not be considered to be highly significant.

### CONCLUSION

This study has shown that the combined effects of pH and concentration of surface-active ions on the reversed-phase liquid chromatographic behavior of weak acids, weak bases, and zwitterionic compounds can be described by a simple ion-interaction model that does not require ion-pair formation in either phase and is not based upon classical ion-exchange.

The simplicity and generality of the mathematical forms of the model make it useful for developing future separation methods.

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