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MOBILE PHASE EFFECTS IN REVERSED-PHASE CHROMATOGRAPHY

II. ACIDIC AMINE PHOSPHATE BUFFERS AS ELUENTS

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SUMMARY

In order to meet the particular requirements with regard to the eluent in liquid chromatography of ionogenic substances on non-polar stationary phases, various buffers composed of phosphate and a vicinal diamine having a pK_a value in acidic pH domain were examined and characterized by their physico-chemical properties. The heat of ionization for such amines is generally much greater than that of acidic compounds having commensurable pK_a values. Consequently changing temperature may strongly affect the degree of ionization of the buffer and as a result the temperature dependence of retention can be drastically altered for certain sample components. This caloric effect of the buffer on retention in reversed-phase chromatograhy was theoretically treated by taking into account the enthalpies for all processes involved, the constants of the protonic equilibria in solution and the limiting retention factors of both the fully ionized and the neutral forms of the eluite. Thus thermal analysis of a chromatographic system containing a buffer in the eluent and ionogenic solutes by using Van 't Hoff plots of chromatographic data may not yield the enthalpy for solute transfer between mobile and stationary phases due to secondary equilibria. Experimental results did not always agree with the theoretical predictions. The aberration was attributed to complex formation between sample molecules and buffer species, that is to a hetaeric effect of the buffer. Some dramatic changes were observed in retention behavior with concomitant improvement in peak shape and chromatographic efficiency upon replacing sodium phosphate by piperazine or tetramethylethylenediamine phosphate in the eluent at pH 6.0. The propitious buffer effect, however, was attributed, at least in part, to masking the silanol groups at the stationary phase surface by the weak amine component of the buffer. The results demonstrate that besides their classical static role to maintain the pH of a solution constant, buffers may play a variety of other roles and affect significantly the properties and efficiency of a chromatographic system.

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INTRODUCTION

Since their introduction in ion-exchange chromatography of nucleic acid constituents in 1967[1], phosphate buffers have played a special and, for that matter, an enigmatic[2] role in high-performance liquid chromatography (HPLC). Whilst all buffers can maintain the pH of the mobile phase constant and can serve as background electrolytes, they are not equally meritorious in chromatography. For a multiplicity of reasons, which have received surprisingly little attention in the chromatographic literature, the chemical nature of the buffer can not only enhance resolution but can also be responsible for poor efficiency, asymmetric peaks and other untoward phenomena. Recently it has been recognized that in a heterogeneous system, such as the column in reversed-phase chromatography (RPC) with bonded phases, the dynamic role of buffers in affecting rate processes can be of importance in addition to the conventional static role of buffers in simply maintaining the pH constant. Yet, most buffers employed in chromatography are those developed by biochemists for use in controlling chemical phenomena in solution.

The control of protonic equilibria mandates the use of buffers in RPC of ionogenic substances[3,4]. An ideal buffer would have the following properties: (i) chemical stability under conditions usually employed in HPLC; (ii) uniform buffering capacity in the pH range between 2 and 8; (iii) optical transparency preferably down to 200 nm; (iv) compatibility with organic solvents used in RPC, especially methanol and acetonitrile; (v) the potential of masking silanol groups of the stationary phase; and (vi) the facility to accelerate rates of proton equilibration.

The second property is desirable since it would eliminate the necessity of using a host of buffers to span this range. Alkali phosphates cover it fairly well but in the pH range between 3.5 and 5.5 they buffer poorly. The third requirement arises from the common use of variable wavelength optical detectors for monitoring column effluent. Carboxylic buffers commonly used in the above pH range, however, absorb light rather strongly below 230 nm. The extensive use of hydro-organic solvents as eluents prompts the fourth requirement: compatibility with organic solvents. Band spreading attributed to eluite interaction with surface silanols and concomitantly asymmetric peaks make the masking of such groups and the suppression of their effect desirable. Similarly, rapid rates of proton transfer are essential to reduce kinetic contributions to band spreading of ionizable eluites.

In this study we examine several buffers composed of phosphate salts of diamines to determine their suitability in RPC by the criteria proposed. The amines have a relatively large enthalpy of ionization which can lead to anomalous retention behavior of ionizable eluites with changes in temperature. Furthermore, complex formation between buffer species and eluites can give rise to changes in retention. Thus the experimental results and the theoretical treatment of the caloric buffer effect should serve as caveat that RPC of partially ionized eluites may not be subject to the "regular" behavior described for relatively simple neutral eluite molecules in the preceding communication[5].

EXPERIMENTAL

The optical density of amine phosphate buffers was investigated by using a Coleman 124D (Perkin-Elmer, Norwalk, Conn., U.S.A.) double-beam spectophoto-

meter and 1 cm quartz cells. Buffer solutions containing 0.1 mol/l of amine were prepared, the pH was adjusted to the pK_a of the amine and with water as the reference the wavelength was scanned until absorbance reached unity. The wavelength where this occurred was defined as the UV cut-off. Compatibility with organic solvents was investigated by dropwise adding the solvent from a buret to 10 ml of buffer under stirring. The cloud point was observed when the light beam from a Spectra-Physics 155 laser in normal direction became visible against a black background.

Unreported pK_a values were determined by potentiometric titration of 10 ml of 0.05 *M* amine with 0.025 *M* HCl. The pH was monitored with an Accumet 420 digital pH meter (Fisher Scientific, Pittsburgh, Pa., U.S.A.). The liquid chromatograph for the effect of temperature was assembled from a Perkin-Elmer (Norwalk, Conn., U.S.A.) Model 1260 pumping system, a Rheodyne (Berkeley, Calif., U.S.A.). Model 71-05 sample injector, a Perkin-Elmer Model LC-65T detector oven, and a Heath-Schlumberger (Benton Harbor, Mich., U.S.A.) Model SR-204 recorder. The 65T oven compartment was modified by the addition of 10 m of 0.010-in. tubing clamped to the metal components of the oven in order to preheat the mobile phase before the injection valve. The 150 × 4.6 mm column was packed with a 5-μm spherical octyl-silica and was a generous gift from L. R. Snyder of Technicon Corporation (Tarrytown, N.Y., U.S.A.). Before changing the eluent the column was washed with a methanol–water mixture containing 30% (v/v) methanol and 1% (v/v) concentrated phosphoric acid. All solutes were made up at a 1 mg/ml level in distilled water and injections of 2 to 10 μl were made after both the detector baseline and a thermometer suspended in the oven compartment stabilized.

The effect of a buffer on efficiency and selectivity was investigated by using a chromatographic system that consisted of an Altex 100A pump (Berkeley, Calif., U.S.A.), a Rheodyne 70-10 injection valve with a 20-μl loop, a Schoeffel 770 detector set to 220 nm (Westwood, N.J., U.S.A.), a Perkin-Elmer 123 recorder and a 250 × 4.6 mm Knauer RP-18 column (Knauer, Berlin, G.F.R.). In some experiments a 10-μm Partisil ODS (Whatman, Clifton, N.J., U.S.A.) column was also employed.

The elution time of an unretained solute, t_o, was measured using fructose as the inert tracer. The retention factor, k, of an eluite was calculated from its retention time, t_R, by using the relationship: $k = (t_R - t_o)/t_o$.

The samples were xanthine, xanthosine and phenylalanine from ICN Pharmaceuticals (Cleveland, Ohio, U.S.A.); adenosine and adenosine 5′-monophosphate from Aldrich (Milwaukee, Wisc., U.S.A.), 4-pyridine carboxaldehyde from Sigma (St. Louis, Mo., U.S.A.); resorcinol, pyridine, phthalic acid, aniline, benzoic acid, and acetone from Chem Service, (Westchester, Pa., U.S.A.) and histidine from Mann Research (New York, N.Y., U.S.A.). Pyridoxine HCl, niacinamide and thiamine HCl were from ICN Pharmaceuticals.

The amines used as buffers included 1,4-diazabicyclo[2,2,2]octane, 1-methyl homopiperazine, piperidine and tropine from Aldrich, methenamine from Fisher (Fairlawn, N.J., U.S.A.), piperazine from Eastman (Rochester, N.Y., U.S.A.) and N,N,N′,N′-tetramethylethylene diamine from Strem Chemicals (Newburyport, Mass., U.S.A.). Cacodylic acid was obtained from Pfaltz and Bauer (Flushing, N.Y., U.S.A.). They were used without further purification.

THEORY

The retention behavior of ionizable substances in RPC depends on the protonic equilibrium between the acid, HA, and its conjugate base, A, and is given by

$$HA \overset{K_1}{\rightleftharpoons} H^+ + A^- \tag{1}$$

where K_1 is the acid dissocation constant of the eluite.
It has been shown[3,4] that in such cases the retention factor can be expressed as a function of the hydrogen ion concentration $[H^+]$ by

$$k = \frac{k_1 + k_2 K_1/[H^+]}{1 + K_1/[H^+]} \tag{2}$$

where k_1 and k_2 are the limiting retention factors of HA and A^-, respectively.

The hydrogen ion activity, *i.e.*, the pH of a given solution, is maintained constant at a given temperature by the action of a buffer which consists of an acid and its conjugate base. The acid is chosen such that its pK_a falls within one unit of the pH value desired. This implies that the ratio of the concentrations of the acid to conjugate base varies between 0.1 and 10. If the temperature is changed, the acid dissociation constant of the buffering compound will be changed and therefore the ratio of the two forms can change. If the buffer concentration is chosen to be much greater than the proton concentration, this effect is minimized and consequently the ratio can be regarded as independent of temperature. The acid dissociation constant of the buffer, K_a, is related to the ratio of the conjugate base and acid forms of the buffering agent, r, and the proton activity, a_H, by

$$K_a = a_H r \tag{3}$$

In this analysis the activity of the proton shall be assumed to be equal to the hydrogen ion concentration, *i.e.*, $a_H = [H^+]$. As a result, eqns. 2 and 3 can be combined to express the retention factor as

$$k = \frac{k_1 + k_2 K_1 r/K_a}{1 + K_1 r/K_a} \tag{4}$$

Whereas r may be assumed to be constant with changing temperature, all other terms on the right-hand side of eqn. 4 depend on the temperature[5] according to the following relationships

$$k_1 = \varphi \exp(-\Delta H_1^0/RT)\exp(\Delta S_1^0/R) \tag{5a}$$

$$k_2 = \varphi \exp(-\Delta H_2^0/RT)\exp(\Delta S_2^0/R) \tag{5b}$$

$$K_1 = \exp(-\Delta H_3^0/RT)\exp(\Delta S_3^0/R) \tag{5c}$$

$$K_a = \exp(-\Delta H_4^0/RT)\exp(\Delta S_4^0/R) \tag{5d}$$

where φ is the phase ratio of the column and ΔH_i^0 and ΔS_i^0 represent the enthalpy and entropy changes appropriate for the particular equilibrium, respectively.

The temperature dependence of the observed capacity factor is given explicitly by combining eqns. 4–5d. The result is given by eqn. 6.

$$k = \varphi \times \frac{\exp(-\Delta H_1^0/RT)\exp(\Delta S_1^0/R) + r\exp\{-(\Delta H_2^0 + \Delta H_3^0 - \Delta H_4^0)/RT\}\exp\{(\Delta S_2^0 + \Delta S_3^0 - \Delta S_4^0)/R\}}{1 + r\exp\{-(\Delta H_3^0 - \Delta H_4^0)/RT\}\exp\{(\Delta S_3^0 - \Delta S_4^0)/R\}} \quad (6)$$

The apparent enthalpy, ΔH_r^0, obtained from Van 't Hoff plots of experimental retention factor data can be expressed by differentiating the natural logarithm of k given in eqn. 6 with respect to the reciprocal temperature as

$$\Delta H_r^0 = \frac{k_1\Delta H_1^0 + (\Delta H_2^0 + \Delta H_3^0 - \Delta H_4^0)\,k_2K_1r/K_a}{k_1 + k_2K_1r/K_a} - \frac{(\Delta H_3^0 - \Delta H_4^0)K_1r/K_a}{1 + K_1r/K_a} \quad (7)$$

In order to show the contribution of each enthalpic term to the total enthalpy observed we may rearrange eqn. 7 with the result that

$$\Delta H_r^0 = \frac{\Delta H_1^0k_1}{k_1 + k_2K_1r/K_a} + \frac{\Delta H_2^0k_2K_1r/K_a}{k_1 + k_2K_1r/K_a} + \frac{(\Delta H_3^0 - \Delta H_4^0)\,(k_2 - k_1)\,K_1r/K_a}{(1 + K_1r/K_a)\,(k_1 + k_2K_1r/K_a)} \quad (8)$$

It is seen from eqn. 8 that in the general case the apparent enthalpy of the chromatographic process is determined not only by the binding enthalpies for the protonated and unprotonated eluite, ΔH_1^0 and ΔH_2^0, respectively, but also on the enthalpy of protonation for both the eluite, ΔH_3^0, and the buffer, ΔH_4^0, in addition to the equilibrium constants involved in the process. As a consequence Van 't Hoff plots of retention data obtained when both forms of the eluite are present to a significant extent in the column are expected to be non-linear.

This can be readily demonstrated for the simple case when the heats of ionization of the buffer and the eluite are identical, *i.e.*, $\Delta H_3^0 = \Delta H_4^0$. In that case the enthalpy is the sum of the binding enthalpies of the two forms, each of which is weighted by the fraction of that species present and its capacity factor. Even in the unlikely event that the binding enthalpies of the two forms are identical, the chromatographic enthalpy will not be constant and the corresponding Van 't Hoff plot will be non-linear.

It is also evident from eqn. 8 that the influence of the buffer ionization is highly dependent upon coupling of that enthalpy to the enthalpy of ionization of the eluite. If they are identical, *i.e.*, $\Delta H_3^0 = \Delta H_4^0$, no "caloric" effect of the buffer will be observed, because the last term in eqn. 8 will be zero. On the other hand, when ΔH_3^0 and ΔH_4^0 are significantly different, the last term can give rise to non-linear Van 't Hoff plots and even to an increase in the retention factor with increasing temperature.

In the limit when the eluite is present only as HA, that is $K_1r/K_a \ll 1$ and $k_2K_1r/K_a \ll k$, or only as A^-, that is, $k_1r/K_a \gg 1$ and $k_2K_1r/K_a \gg k_1$, linear Van 't Hoff plots are obtained according to eqn. 8 and the measured enthalpies ΔH_1^0 and ΔH_2^0 are those for the binding of the undissociated and dissociated acid by the stationary phase, respectively. Eqn. 8 clearly shows that the enthalpy evaluated from retention data,

ΔH^0_r, may be a complex function of the operating conditions even in the simple case where no specific eluite–buffer interactions are postulated. It is therefore conveniently considered as a chromatographic parameter which may be called retention enthalpy.

RESULTS AND DISCUSSION

Buffering properties of amine phosphates

Most siliceous bonded phases employed in HPLC are unstable in contact with aqueous eluents outside of the pH range of 2 to 8, so that buffers in this pH domain are of interest to us. The two pK_a values of phosphate, 2.1 and 7.2, are at the fringes of this range. Therefore, phosphates of strong bases do not have adequate buffering capacity at intermediate pH values.

It was thought that weak bases having a pK_a value in the vicinity of 4 could beget phosphate buffers of substantial buffering capacity over the whole pH span. According to Table I, which lists the pK_a value and enthalpy of ionization for a wide range of ionogenic substances, vicinal diamines are the most promising candidates. The low pK_a value is due to the repression of the ionization of the second amino group by the proximity of a cationic site.

TABLE I

RANGES OF pK_a AND ΔH^0 OF IONIZATION FOR DIFFERENT CLASSES OF POTENTIAL BUFFERS AND ELUITES

Buffer type	*pK_a*	*ΔH^0 (kcal/mol)*	*Reference*
Inorganic acids	2–6.5	−1.8 to 1	6
Carboxylic acids	2.8–5.6	−1 to 0.7	6
Amines			
primary	9.8–10.6	10	6
secondary	4.2–10.8		
tertiary	4.8–9.8		
Vicinal diamines	3–6	4–10	7
α-Amino acids			
carboxyl group	2.1–2.4	1	6
amino group	9.7–9.8	10	6

In order to meet the requirement of optical transparency at low short wavelengths the amine molecule should not have an aromatic moiety or another chromophore. Table II shows a list of selected amines for potential use with phosphate as a buffer in acidic eluents for RPC. Fig. 1 shows the pH ranges over which the phosphate salts of the amines listed in Table II would give effective buffers on the basis of their pK_a values.

The pH dependence of the buffering capacity of such a typical prospective amine phosphate buffer is illustrated in Fig. 2. The dependence of the buffer capacity, β, on pH has been calculated by the formula given by Butler[9] for diprotic substances as

$$\beta = 2303\, C\, K_{a1}\, [H^+]\, \frac{(1 + K_{a1}/K_{a2})[H^+]^2 + 4\, K_{a2}[H^+] + (1 + K_{a2}/K_{a1})K_{a1}K_{a2}}{([H^+]^2 + (1 + K_{a2}/K_{a1})K_{a1}[H^+] + K_{a1}K_{a2})^2} \quad (9)$$

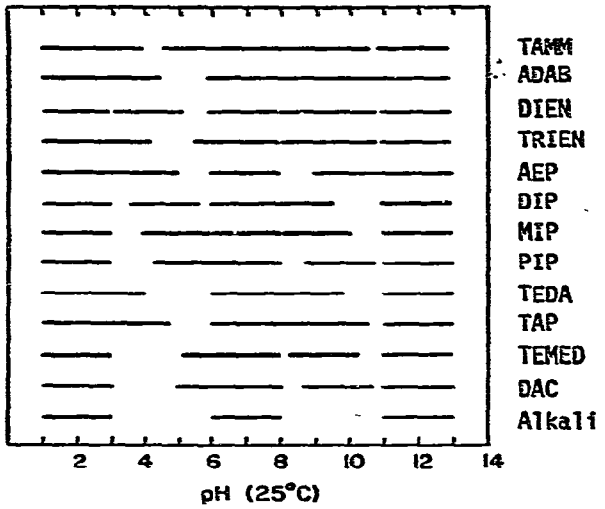


Fig. 1. Buffering range of amine phosphates. The ranges were taken as 1 pH unit above and below the acidic pK_a value of the amine. The buffering ranges of sodium phosphate are also shown for comparison. The meanings of the acronyms for amine components are given in Table II.

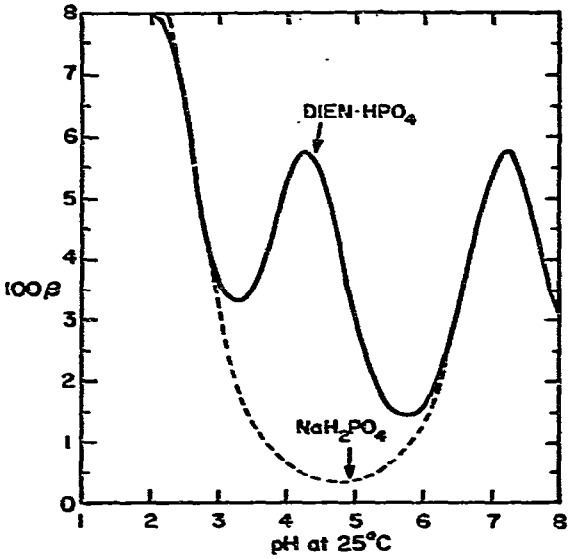


Fig. 2. Buffering capacity, β, of 0.1 *M* DIEN phosphate solutions (solid) and 0.1 *M* sodium phosphate solutions (dashed) as a function of pH at 25°C.

where C is the buffer concentration, and K_{a1} and K_{a2} are the larger and smaller ionization constants, respectively. It is seen from Fig. 2 that a 0.1 *M* solution of diethylenetriamine phosphate is markedly superior to phosphate solutions in terms of β in the pH range between 3.5 and 5.5 where phosphate buffers poorly.

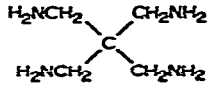
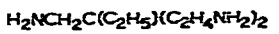
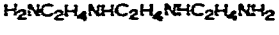
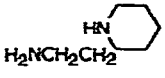
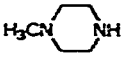
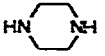
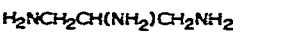
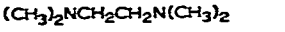
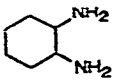
Physical properties of the prospective buffer substances

As the most popular detection mode in HPLC today is photometric at low

TABLE II

ACRONYM, STRUCTURE, pK_a AND HEAT OF IONIZATION FOR AMINES SHOWING BUFFER POTENTIAL IN THE ACID pH RANGE

All pK and $\Delta H°$ values were determined at 25°C at an ionic strength (μ) of 0.1 unless otherwise noted.

Abbreviation	*Amine*	*Structure*	pK_a	$\Delta H°$ *(kcal/mol)*	*Ref.*
TAMM	Tetrakis(amino methyl)methane		3.03 5.67 8.17 9.89		8
ADAB	1-Amino-2-di(amino ethyl)butane		4.25 8.98 9.78	7.2 12 11.2	7
DIEN	Diethylenetriamine		4.23 9.02 9.84	7.2 12 11.2	8
TRIEN	Triethylenetetra-amine		3.25 6.56 9.08 9.74	4 7 9.4 10	8
AEP	2-(2-Aminoethyl) piperidine		3.94* 10.03	3.77* 11.4	7
DIP	1,4-Dimethyl-piperazine		4.63** 8.53	4.42** 6.21	7
MIP	1-Methylpiperazine		4.94 9.09	4.0 8.4	7
PIP	Piperazine		5.76 9.72	6.9 10.2	7
TEDA	Triethylene diamine		2.97 8.82	3.01 7.3	7
TAP	1,2,3-Triamino-propane		3.72*** 7.95 9.59		8
TEMED	N,N,N′,N′-Tetra-methylethylene diamine		6.13§ 9.28	6.64§ 7.4	8
DAC	1,2-Diamino-cyclohexane		6.0* 9.6	10.1* 11.6	7

* $\mu = 0$.
** $\mu = 1$.
*** $\mu = 0.5$.
§ $T = 20°C$.

wavelengths, it is important that the mobile phase be transparent to UV detectors. Sodium and potassium phosphate have been a standard buffer in HPLC. Their transparence extends to 200 nm, whereas carboxylic acid buffers, which have been widely used for the pH 3 to 6 range, have a relatively high cut-off value.

The saturated amino-cations selected for this study can reasonably be expected to be transparent at low wavelengths. The ultraviolet absorbance of several 0.05 *M* amine phosphate buffers was examined and the cut-off value, *i.e.*, the wavelength at which the absorbance is 1.0, was determined. The results are recorded in Table III, which shows that most of the phosphate buffers tested are optically clear even at relatively low wavelengths. Since all of the materials were "reagent" or "practical" grade and used without any further purification, it is possible that their cut-off value can be reduced upon additional cleanup. Some of the polyamines such as DIEN and TRIEN, however, are readily oxidized so that their transparency may decrease with time.

The miscibility of these buffers with methanol and acetonitrile was examined and the results are presented in Table III. The amines were quite soluble in water, methanol and acetonitrile. However, their phosphate salts showed relatively poor solubility in organic solvents. Solvation by the alcohols, however, is sufficient to allow the preparation of adequate hydro-organic eluents and gradient operation with most of these systems. Just as sodium phosphate has a lower solubility in acetonitrile than in alcohols, these new amine phosphate buffers also exhibit a lower compatibility with acetonitrile. Since acetonitrile is such a popular HPLC solvent, we are investigating the preparation of amine buffers having higher solubility due to the employment of anions different from phosphate. As an alternative the potential of more hydrophobic buffer amines such as N,N'-dimethylpiperazine is under study.

TABLE III

PROPERTIES OF ACIDIC BUFFERS, INCLUDING THE NEW AMINE PHOSPHATES, OF CHROMATOGRAPHIC INTEREST

The pK_a and ΔH values appropiate at 25°C and the ionic strength are indicated. The cut-off wavelength, at which the absorbance against water is unity, was determined for an aqueous 0.05 *M* solution of buffer. The miscibility with acetonitrile and methanol was determined by the cloud point given by that volume fraction of organic component which in a mixture with a 0.05 *M* buffer formed a cloud. The pK_a and ΔH are obtained from the sources shown by the reference unless otherwise indicated.

Buffer	μ	pK_a	ΔH° *(kcal/mol)*	*Ref.*	*pH*	*Cut-off 1 cm (nm)*	*Methanol cloud point*	*Acetonitrile cloud point*
Sodium phosphate	0	2.1	−0.8	7	2.21	195	>0.99	0.85
		7.2	0.7		6.0	195	>0.99	0.73
PIP phosphate	0	5.33	7.42	7	4.37	195	0.55	0.22
					6.0	195	0.48	0.47
1-Methyl-homopiperazine phosphate	0*	6.1		—	4.35	210	0.92	0.65
DIEN phosphate	0.1	9.84	11.2					
		9.02	12.0	6				
		4.23	7.2	—	4.20	213	0.44	0.37
TEMED phosphate	0.5	6.13	6.64					
		9.28	7.4	6	6.0	228	>0.99	0.22
Sodium acetate	0	4.76	−0.1	7	4.2		>0.99	>0.99
Hexamethylenetetramine phosphate	0*	4.8**	—	—	4.4	220	>0.99	0.85
Sodium cacodylate	0	6.2	0	10	6.0	—	—	—

* $T = 21$°C.

** Obtained by a potentiometric titration with 0.025 *M* HCl.

Temperature effects

Since almost all buffers and solutes encountered in liquid chromatography have a non-zero heat of ionization, their pK_a values are temperature dependent. In the case of acids, the heat of ionization is close to zero, see Table I, and the effect is small. For phosphate, for instance, ΔH^0 is about −1 kcal/mole so that the pK_a value ranges from 2.12 at 15°C to 2.25 at 45°C. On the other hand for a basic solute such as aniline, ΔH^0 is significantly higher, of the order of 7 kcal/mol. Therefore, its pK_a will range from 4.78 at 15°C to 4.27 at 45°C[5]. In a chromatographic system with the eluent pH close to the pK_a's of the buffer and solute the dependence of ionization on temperature might be used to develop and improve separations involving ionogenic solutes by proper adjustment of the temperature.

As described before in conjunction with eqn. 8, if the heats of ionization are identical for both the buffer and solute, or if the pK_a of the solute is distant from that of the buffer, then no special effects would be expected. However, if the pK_a of the solute is near the buffer pH, and if their respective heat of ionization differ significantly, then a special dependence of the retention on the temperature would be anticipated.

In order to view this effect, amine phosphate buffers, which are shown in Table II and have a range of ΔH^0 values from −0.8 to 7.4 kcal/mole, as well as the solutes listed with their properties in Table IV and have a similar range of enthalpies, were examined experimentally. Most of the solutes yielded linear Van 't Hoff plots which allowed the evaluation of the retention enthalpies presented in Table IV. As expected, retention enthalpies were dependent upon the buffer used and for all solutes with pK_a's near the eluent pH retention was more strongly dependent on the nature of the buffer than for those with distant pK_a's. Resorcinol, with a pK_a 3.3 units higher than the pH of the buffer, showed a surprising change in retention enthalpy which could be due to formation of a weak hydrogen-bonded complex with the diamine.

TABLE IV

RETENTION ENTHALPIES MEASURED WITH PLAIN AQUEOUS SODIUM AND AMINE PHOSPHATE BUFFERS, pH 6.00 AT 25°C, ON OCTYL-SILICA IN THE TEMPERATURE RANGE 30 TO 90°C

The pK_a and enthalpies are taken at 0.1 ionic strength and 25°C unless otherwise noted.

Solute	*pK_a**	*ΔH^0_{ion} (kcal/mol)*	*Ref.*	*$-\Delta H^0_r$ (kcal/mol)*		
				Sodium phosphate	*Piperazine phosphate*	*TEMED phosphate*
Aniline	4.6	7.28	7	1.34	1.80	1.77
Benzoic acid	4.2	0.1	7	1.58	1.86	1.50
Resorcinol	9.3	—	8	1.99	3.05	2.63
Niacinamide	3.5	—	8	2.02	2.77	2.72
Xanthosine	5.7	3.74	7	2.00**	5.19	3.35
Xanthine	7.5	6.33	7	3.87	4.06	2.47
AMP	6.2	4.2	7	2.78	3.35	1.50**
Phenylalanine	2.9	0.6	7	1.47	2.45	1.89

* The pK_a value closest to 6.0 when there is more than one ionogenic group in the solute molecule.

** Van 't Hoff plot was non-linear, see Fig. 3. The values shown are estimated from the plot at 333°K.

The retention of adenosine monophosphate, phthalic acid and xanthosine has shown strong dependence on both the buffer type and the temperature as illustrated in Fig. 3. The parameters used for calculating the curves from eqn. 6 were taken for each compound from the literature or from Van 't Hoff plots of data obtained in different sets of experiments. The values of k_1 and ΔH_1^0, the retention factor and the binding enthalpy, respectively, for the lower end point were evaluated in pH 2.2 sodium phosphate or pH 4.2 sodium acetate. The k_2 and ΔH_2^0 values, which are limiting values at high pH, were measured either in pH 6.0 sodium phosphate or obtained by extrapolation.

It is seen from Fig. 3 that experimental data obtained by using sodium phosphate buffers are in good agreement with the predicted behavior. For the amine phosphate buffers, however, the theory of the caloric effect did not correctly predict the retention factor for the eluites upon changing temperature. However, the slopes and concomitantly the retention enthalpies did agree with the predicted values at higher temperatures.

The discrepancy suggests a strong interaction between the solute and buffer molecules in addition to the pH and caloric effects. This tertiary action of the buffer represents an additional equilibrium phenomena and it may be similar to ion-pair formation that was denoted a hetaeric effect[11]. It is most pronounced in the TEMED phosphate system; the piperazine system shows better agreement with the anticipated behavior. Both amines used in the buffers are diamines and have a fully protonated amine group at the eluent pH already. The degree of ionization for the second amine group of TEMED (pK_a 6.13) is greater than that of piperazine (pK_a 5.33) at pH 6.0. The stronger hetaeric effect found with TEMED would therefore be expected.

The very large deviation from the predicted behavior at low temperature indicates that there may be another buffer–solute interaction that becomes negligible at temperatures above 50°C, because it has a very large enthalpy change. This secondary hetaeric effect is strongest with the dibasic phthalic acid but it can be seen with the other two solutes also. One hetaeric effect may be caused by buffer molecules adsorbed by the surface of the stationary phase due to combined hydrophobic and electrostatic binding forces, whereas the other hetaeric effect may be due to secondary equilibria that occur in the eluent proper.

Combined caloric and hetaeric effects may dramatically alter the selectivity of a chromatographic system as illustrated in Fig. 4 which shows the reversal of elution order on chromatograms obtained at different temperatures. In the case illustrated the inversion is due to the low retention enthalpy of phthalic acid with its decreased ionization with temperature as opposed to the normal behavior exhibited by resorcinol. In the temperature range investigated the pK_a of phthalic acid increases from 5.4 to 5.6, while the pH of the buffer drops from 6.0 to approximately 5.4. As a result, the dissociation of the acid is suppressed and it is retained longer as the column temperature rises. Further studies are planned to clarify the mechanisms of the other phenomena involved in these systems.

Buffer selectivity effects

Eluents containing amine phosphate buffers manifest chromatographic selectivities different from those shown by sodium phosphate buffers under otherwise identical conditions. A column packed with octyl-silica, which had been exhaustively

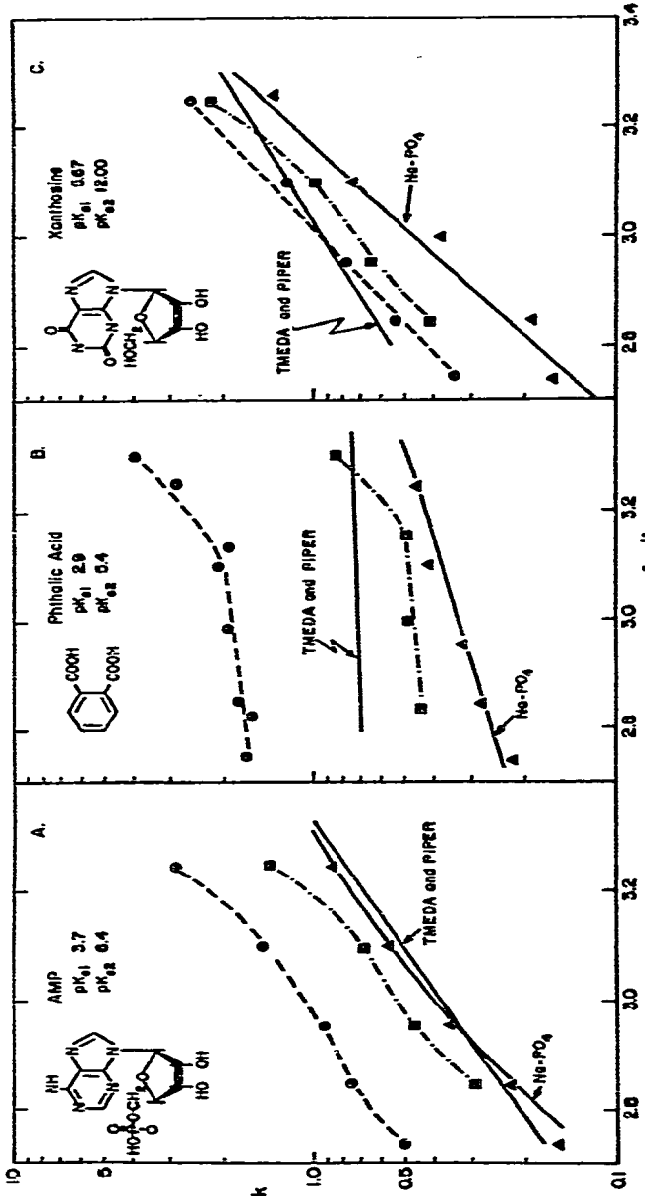


Fig. 3. Graphs illustrating theoretical (solid lines) and experimental Van 't Hoff plots of retention data calculated for or obtained with AMP, phthalic acid and xanthosine on octyl-silica by using sodium phosphate(▲) piperazine phosphate(■) or tetramethylethylenediamine phosphate(●). In all calculations eqns. 4 and 5 were used and the heat of ionization at pH 6.00 was taken as −1.0 and 7.0 kcal/mol for sodium and amine phosphate, respectively. The parameter values for the eluite were taken from the literature and recent experimental data where the value of pK_a, k_1 and k_2 are given at 25° and the enthalpies are in cal/mol: AMP, $pK_a = 6.2$, $k_1 = 0.75$, $k_2 = 1.2$, $\Delta H_1 = \Delta H_2 = -3000$, $\Delta H_3 = -1100$; phthalic acid, $pK_a = 5.4$, $k_1 = 3.0$, $k_2 = 0.01$, $\Delta H_1 = \Delta H_2 = -2000$, $\Delta H_3 = -600$; xanthosine, $pK_a = 5.67$, $k_1 = 5.2$, $k_2 = 0.4$, $\Delta H_1 = \Delta H_2 = -3500$, $\Delta H_3 = -3700$.

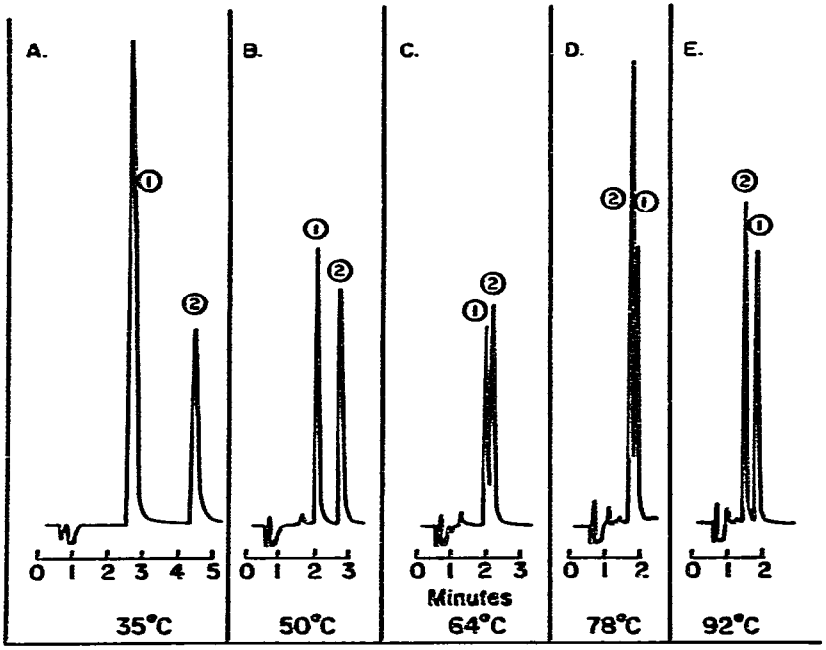


Fig. 4. Illustration of the inversion in elution order with increasing temperature due to the strong caloric effect of the amine phosphate buffers on the retention of phthalic acid (1) but not on that of resorcinol (2) on octyl-silica. Mobile phase, 0.05 *M* TEMED phosphate pH 6.00; flow-rate, 2 ml/min; UV detection at 220 nm.

trimethylsilylated to eliminate all free silanol groups at the surface, showed an enhancement of the retention factors for all compounds when the amine phosphate buffers replaced sodium phosphate buffer as seen from the results in Table V and in Fig. 5 as well. The largest increase in retention modulus*, η, which is defined here as the retention factor in amine phosphate buffer divided by that in sodium phosphate buffer under otherwise identical conditions, occurred with carboxylic acids, while amines and amides showed the smallest enhancement in retention factor. The enhancement of retention is attributed to the strong hetaeric effects of the amine phosphate buffers that was cited in the preceding section.

Another example for the modification of retention factors by buffers can be seen in Table VI in which the retention moduli with sodium phosphate as the reference buffer are shown for three different columns and two buffers. The large decrease in the retention factors of the thiamine that occurs when the sodium phosphate is replaced by an amine phosphate buffer is particularly noteworthy. In view of the high k value for thiamine on Partisil 10 ODS, the surface of which is only partially covered by hydrocarbonaceous functions, suggests that these strongly basic eluites are not retained

* In a recent communication[12] the notion of retention modulus was introduced to express a relative change in the retention factor of an eluite upon changing the mobile phase under otherwise invariant conditions. The modulus, η, is particularly suited to express the retention modulating effect of a solvent or more importantly secondary equilibria between the eluite and a complexing agent (modulator or hetaeron) added to the eluent.

TABLE V

EFFECT OF REPLACEMENT OF SODIUM PHOSPHATE BY AMINE PHOSPHATE BUFFERS IN THE ELUENT ON SOLUTE RETENTION ON DIFFERENT HYDROCARBONACEOUS BONDED PHASES

All buffers were 50 m*M*, pH 6.00 at 25°C; column temperature was 21°C. The carbon load of the Partisil ODS (P-ODS) was 5.2% carbon and LiChrosorb RP-18 (RP-18) contained 22% carbon. The carbon content of the octyl-silica (RP-8) is unknown.

Solute	*Retention factor with sodium phosphate*			*Modulus, η*				
				Piperazine phosphate		*TEMED phosphate*		
	P-ODS	*RP-18*	*RP-8**	*P-ODS*	*RP-18*	*P-ODS*	*RP-18*	*RP-8**
Pyridoxine	3.2	5.0	2.77	0.97	0.94	0.58	0.94	0.94
Niacinamide	7.0	9.3	3.64	1.16	0.89	0.60	0.86	1.04
Thiamine	69.0	13	8.85	0.49	0.75	0.11	0.36	0.30

* Octyl-silica column was operated at 35°C.

by a simple hydrophobic mechanism, but may interact with free silanol groups at the surface of the stationary phase as well. A potential value of these buffers rests also with the masking of accessible polar groups, particularly acidic groups like silanols, which may be present. Masking surface silanol groups and the concomitant tailing reduction by amine phosphate buffers may be responsible for the improvement in the analytical results illustrated by the chromatograms of the three B vitamins in Figs. 6 and 7. The untoward chromatographic effect of free silanols is expected to be great

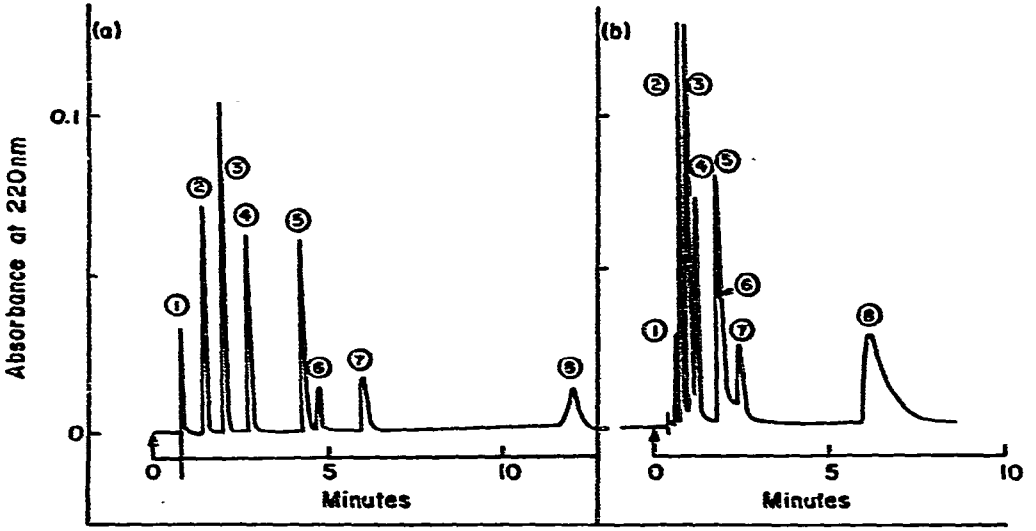


Fig. 5. Comparison of chromatograms obtained with sodium phosphate and piperazine phosphate buffers. (a) 0.05 *M* piperazine phosphate pH 6.00; (b) 0.05 *M* sodium phosphate pH 6.00. Column, 5 μm octyl-silica, 150 × 4.6 mm; temperature, 30°C; flow-rate, 2 ml/min. The sample components are: (1) histidine, (2) phthalic acid, (3) xanthine, (4) xanthosine, (5) adenine phosphate, (6) phenylalanine, (7) resorcinol and (8) aniline.

TABLE VI

EFFECT OF THE REPLACEMENT OF SODIUM BY AN AMINE CATION IN PHOSPHATE BUFFERS ON RETENTION

The retention factor obtained in sodium phosphate buffer and the retention moduli, η, obtained with piperazine phosphate and TEMED phosphate buffers are given. The measurements were carried out on octyl-silica with 50 m*M* buffers, pH 6.00 at 35°C.

Solute	*Sodium phosphate, k*	*Piperazine phosphate, η*	*TEMED phosphate, η*
4-Aminosalicylic acid	0.52	1.56	4.31
Phthalic acid	0.52	1.40	5.42
Adenosine monophosphate	0.88	1.61	3.20
Xanthine	0.93	1.29	1.45
Xanthosine	1.25	1.60	2.08
Acetone	1.60	1.00	1.31
Benzoic acid	2.85	1.58	3.51
Phenylalanine	3.49	1.20	1.08
Niacinamide	3.64	1.28	1.04
Resorcinol	4.50	1.13	1.23
Aniline	10.2	1.18	1.07

when the surface coverage with the hydrocarbonaceous ligates is low, such as in the octadecyl-silica Partisil 10 ODS which has a carbon load of 5–6% Indeed a greater improvement in the chromatographic results upon changing from sodium phosphate

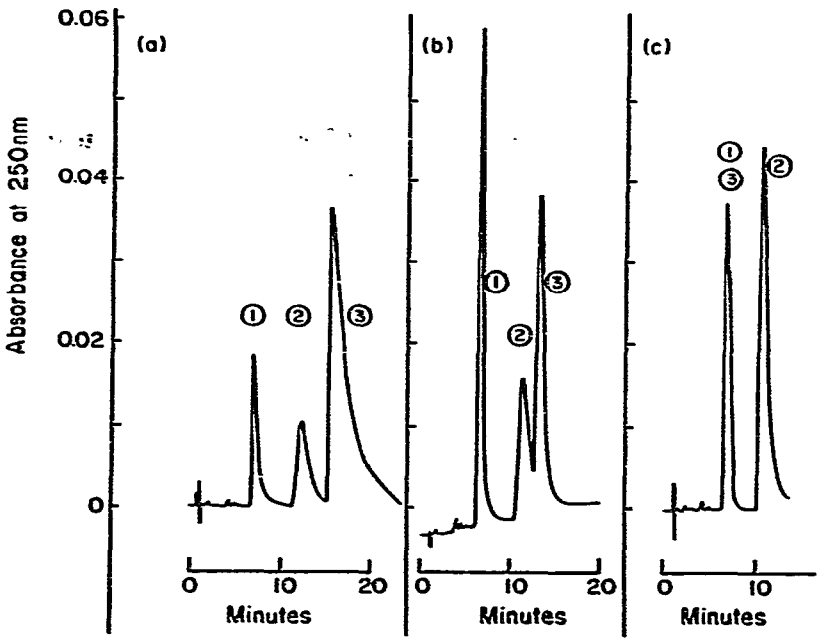


Fig. 6. Illustration of chromatograms obtained with the three B-vitamins by using different mobile phases: (a) 0.05 *M* sodium phosphate, pH 6.00; (b) 0.05 *M* piperazine phosphate, pH 6.00; and (c) 0.05 *M* tetramethylethylenediamine phosphate, pH 6.00. The sample components are: (1) pyridoxine, (2) niacinamide and (3) thiamine. The chromatograms were obtained with a Knauer LiChrosorb RP-18 column at 20°C with a flow-rate of 2 ml/min.

to amine phosphate in the eluent can be noted with the Partisil 10 ODS column, see Fig. 7, than with the LiChrosorb RP 18 column, *cf.* Fig. 6, in which the surface coverage of octadecyl silica is significantly higher.

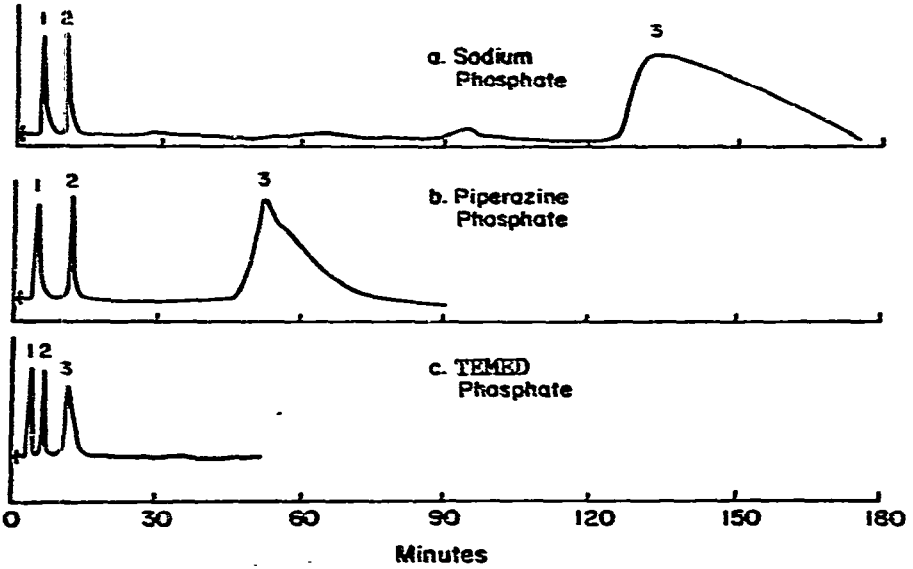


Fig. 7. Effect of buffer on the chromatographic separation of (1) pyridoxine, (2) niacinamide and (3) thiamine on octadecyl-silica of low surface coverage. Column, Partisil 10 ODS, 250 × 4.6 mm (5% carbon load); eluent, 0.05 *M* buffer, pH 6.00 at 25°C; flow-rate, 2 ml/min; temperature, 22°C; UV detection at 250 nm.

Examination of the chromatograms in Figs. 6 and 7 reveal that the retention factors of pyridoxine did not change significantly upon changing from sodium phosphate to amine phosphate buffer in the eluent. Yet, the peaks became sharper and more symmetrical with a concomitant improvement in resolution. This phenomenon may be attributed to a dynamic effect of the buffer[13,14] in accelerating protonic equilibria involved in the chromatographic process.

CONCLUSIONS

(1) The development of novel buffers to meet particular needs of reversed phase chromatography was attempted by using phosphates of vicinal diamines.

(2) A theoretical model was introduced to describe the effect of temperature on the retention of ionizable substances as a function of the enthalpies of binding to the stationary phase surface for the neutral and fully ionized eluites, the heats of ionization for the eluite and buffer in solution and the corresponding four equilibrium constants.

(3) The theory predicts and experimental data show that in a chromatographic system operated at an eluent pH close to the pK_a values of both the buffer and the eluite, Van 't Hoff plots of the retention factors may not yield straight lines depending on the relative magnitude of the pertinent enthalpy changes. In fact, under certain conditions the theory predicts increasing retention with temperature.

(4) The so-called caloric effect of the buffer can dramatically alter the selectivity of the chromatographic system upon changing column temperature.

(5) Analysis of chromatographic data obtained at different temperatures within the framework of the theoretical model revealed that complex formation (ion pairing) between eluites and the buffer also occurs.

(6) The hetaeric effect of the buffer is also demonstrated by comparing retention moduli obtained with amine phosphate buffers using sodium phosphate as the reference.

(7) The results suggest that the amine component of amine phosphate buffers interact with silanol groups at the stationary phase surface. The attendant masking of the silanols may be responsible for the reduced tailing and improved resolution observed with positively charged sample components in particular.

(8) The investigation showed that besides their conventional static and dynamic role, buffers can manifest caloric and hetaeric effects of chromatographic importance.

(9) Further search for improved buffers and for an understanding of their higher order chromatographic effects is an essential part of eluent engineering to exploit the full potential of non-polar stationary phases in HPLC.

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