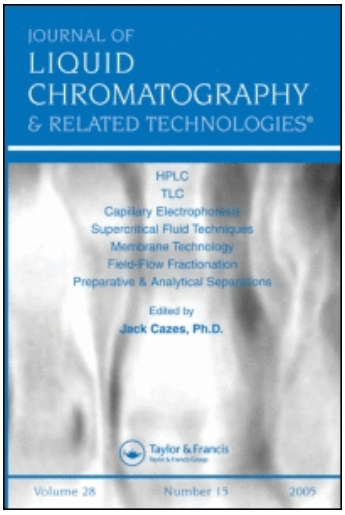

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INFLUENCE OF EXPERIMENTAL PARAMETERS ON CHROMATOGRAPHIC BEHAVIOR OF NEUTRAL MOLECULES IN ION INTERACTION CHROMATOGRAPHY

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ABSTRACT

The chromatographic behavior of aprotic neutral molecules in Ion Interaction Chromatography (IIC) was investigated both theoretically and experimentally on a styrene-divinylbenzene based C_{18} stationary phase. The chemical modification of the stationary phase in the presence of Ion Interaction Reagent (IIR) in the eluent, and adsorption competition between tested analytes and IIR for inner layer sites are theoretically shown to change the partition coefficient for neutral molecules.

Experimental evidence confirms that their retention decreases with increasing ion-interaction reagent (IIR) concentration in the eluent. The influence of the mobile phase ionic strength on neutral molecules retention was investigated and the dependence of retention modulus on organic modifier concentration in the eluent was elucidated.

INTRODUCTION

The Ion Interaction Chromatography of neutral molecules seems to be a forgotten field for separation scientists.[1] While the effect of ion interaction reagents on the retention factor (k) values of oppositely charged analytes has been well studied,[2-7] the influence of ion-interaction reagents on the k values of neutral species has not been widely investigated.

Little has been done to understand IIC of neutral molecules, and a wide variability among experimental results can be found, even if it is usually accepted that their retention does not depend on the IIR concentration in the eluent.[2] This prompted us[1] to investigate the chromatographic behavior of neutral molecules by taking into account the exhaustive retention model of Bidlingmeyer.[4-5] This is broader in scope than either a dynamic ion-exchange mechanism[8-10] or an ion-pair mechanism,[11-13] since retention of the sample is viewed as a transfer through the electrical double layer which develops at the stationary phase surface if lipophilic ions of the IIR are present in the eluent. Hence, retention results from both electrical and van der Waals forces.

Test compounds were intentionally selected[1] to cover the chemical and physical properties which are supposed to be a requisite, on the basis of the Ion Interaction Model premises, for competition for the inner layer sites on the stationary phase surface. Since the driving force for the adsorption of the IIR onto the stationary phase is the high surface tension which is generated between the non polar stationary phase and the polar mobile phase, polar organic neutral molecules are easily predicted to compete with lipophilic ions[3] for inner layer sites on the stationary phase. Taking this into account, we decided to investigate the chromatographic behavior of neutral analytes and to study the influence of experimental parameters on their chromatography.

Selected experimental conditions were designed to deconvolve silanophilic from dispersion and electrostatic interactions and to pinpoint the exact source of the observed phenomenon. The use of a styrene-divinylbenzene based C_{18} stationary phase, whose base material is itself strongly hydrophobic, and displays no ionic character whatsoever proved to be[1] a valuable means to deconvolve the electrostatic interactions of tetraalkylammonim salts with residual silanol groups[14-15] from pure ion interactions.

Herein, we have theoretically shown that factors affecting solute retention are amenable to theoretical treatment. Moreover, the influence of the mobile phase ionic strength on neutral molecules retention was investigated and the dependence of retention modulus on organic modifier concentration in the eluent was elucidated.

EXPERIMENTAL

A 1090 series II Hewlett Packard (Palo Alto, CA, USA) high pressure liquid chromatograph with factory supplied diode array detector and variable volume 25-µL syringe based auto-injector (Rheodyne sample injection valve Model 7010) was used. The analyses were run at room temperature under isocratic elution condition. The detector was operated at 254 nm for detection of acetone (DMC) and 210 nm for detection of dimethylformamide (DMF), tetramethylurea (TMU). The column was oven thermostatted at 28°C.

All experiments were carried out with a 15 cm x 4.6 mm I.D containing 9 µm Polyspher (C_{18} functionalized SDVB copolymers particles) C_{18}, 120Å pore diameter, stainless steel column, purchased from Merck. The eluent flow-rate was 0.700 mL/min.

The hold-up time (t_o) was determined by injecting 25 µL of water and measuring the time from injection to the first deviation from the baseline. Tetrabutylammonium dihydrogen phosphate, dimethylformamide (DMF), tetramethylurea (TMU), and acetone (DMC) were purchased from Aldrich (Milwaukee, WI, USA); potassium dihydrogen phosphate and disodium monohydrogen phosphate were purchased from Merck (Darmstadt, Germany); all chemicals were of the best available quality and used without further purification. Water was produced by a Milli-Q 185 system (Millipore, Bedford, MA, USA).

The eluent systems are detailed in the captions. All solutions were filtered through a 0.45 µm pore size regenerated cellulose filter (Schleicher & Schuell, Dassel, Germany).

Injection volumes were 0.1µL for DMF 9.4 g/L, 2 µL for DMC 7.9 g/L and 1 µL for TMU 9.7 g/L. Injection volumes were selected so that any further decrease of sample size did not change analyte retention when IIR was not present in the mobile phase. Analytes were filtered through a 0.2 µm pore size nylon filter (Lida, Kenosha, WI, USA).

Prior to use, the reversed phase columns were equilibrated with the solvent system to be used for 1 h at a flow rate of 0.700 mL/min. Equilibration was established by obtaining similar results in duplicate runs at a 15 min. interval.

THEORY

In order to shed light on the relationship between the capacity factor of neutral molecules and the IIR concentration in the eluent, the process first will

be treated phenomenologically. The binding of the eluite and the IIR with the stationary phase is obviously supposed to take place independently since no electrostatic interaction can occur between them in the mobile phase. For the reversed phase chromatography of a neutral molecule the following equilibrium can be written:

$$L+E \overset{K1}{\rightleftharpoons} LE \tag{1}$$

where L is the accessible hydrocarbonaceous ligand, and E represents the eluite. For the binding of the IIR we have:

$$L+H \overset{K2}{\rightleftharpoons} LH \tag{2}$$

where H is the hydrophobic IIR. For the equilibrium constants it holds:

$$K_1 = [LE]_s / [L]_s \cdot [E]_m \tag{3}$$

$$K_2 = [LH]_s / [L]_s \cdot [H]_m \tag{4}$$

where concentrations in the mobile and stationary phase are denoted by the subscripts m and s respectively. The IIR concentration in the eluent can be considered constant since the eluite can not directly form any complex with it. Hence we may write:

$$[H]_m = [H] \tag{5}$$

The capacity factor of the neutral molecule, k, is defined in the usual way as:

$$k = \Phi \cdot [LE]_s / [E]_m \tag{6}$$

where Φ is the phase ratio of the column. The combination of Equation (3) and (6) yields

$$k = \Phi \cdot K_1 \cdot [L]_s \tag{7}$$

Since the extent of binding of the eluite with the stationary phase accessible sites is expected to be small and the total ligand concentration $[L]_T$ is conserved we are allowed to write:

$$[L]_T = [L]s + [LH]\ s \quad (8)$$

By substitution of Equation (8) into Equation (7) we have:

$$k = \Phi \cdot K_1 \cdot ([L]_T - [LH]_S) \quad (9)$$

Since the amount of the adsorbed hydrophobic ions $[LH]_S$ is related to the concentration of the IIR in the mobile phase by an adsorption isotherm, we may write:

$$[LH]_S = f\{[H]\} \quad (10)$$

where the explicit form of the function f can change according to the kind of stationary phase and hydrophobic reagent used.

By substitution of Equation (10) into Equation (9) we have:

$$k = \Phi \cdot K_1 \cdot ([L]_T - f\{[H]\}) \quad (11)$$

hence the capacity factor of a neutral molecule is easily predicted to decrease with increasing IIR concentration in the eluent because of adsorption competition.

However, surface exclusion phenomena must not be viewed as the only cause of the observed dependence of neutral molecules retention upon IIR concentration.

We want to underline that the equilibrium constant for the partition of a neutral analyte between the stationary and the mobile phase, K_1, can not be considered constant after the addition of the IIR in the mobile phase.

According to solvophobic theory:[16]

$$\ln K1 = a + b\gamma \quad (12)$$

it is clear that the interfacial tension γ can not be viewed as a constant if the IIR concentration in the eluent is varied. Moreover, the parameters a and b depend on the column properties and eluent composition which would both be altered after the addition of the IIR in the eluent.

It has also to be taken into account that, even if test substances are not charged compounds, they can interact with the stationary phase, also, via their electrical dipolar moment, according to the stationary phase surface charge density, which in turn depends on the IIR concentration in the mobile phase.

For these reasons K_1 must be considered a function (f ') of the lipophilic ion concentration in the eluent, hence the capacity factor can be expressed by:

$$k = \Phi \cdot f'\{[H]\} \cdot ([L]_T - f\{[H]\}) \quad (13)$$

from which it is evident that the course or neutral molecules retention upon IIR concentration in the eluent can be very complex and variable.

RESULTS AND DISCUSSION

The attenuation in retention upon IIR presence in the eluent[1] is conveniently expressed by the modulus, η, that is defined by:

$$\eta = k_0/k \quad (14)$$

where k and k_0 are the retention factors of the tested analyte with and without the IIR in the eluent both measured under otherwise identical conditions. This parameter is very helpful because it enables one to deconvolve the influence of experimental parameter on analyte retention from that on analyte retention attenuation. Moreover the value of the modulus for the highest IIR concentration (η_{25}) can shed light on the physico-chemical basis of the IIC of neutral molecules.

Table 1 and Table 2 show the effect of methanol concentration in the eluent on the retention modulus of uncharged solutes respectively in buffered and aqueous mobile phase. In each case η decreases in a regular manner with increasing methanol concentration.

It is clear that the value of the attenuation modulus η_{25} is influenced by the presence of methanol concentration in the eluent. For molecules such as benzene and toluene, which are not supposed to be able to penetrate the inner layer at the stationary phase surface, an accurate analysis of the reported literature data[17] evidences that the attenuation of their retention, upon the presence of a fixed concentration of IIR in the eluent, does not depend on methanol concentration, even if retention does.

For surface active test substances, not only retention, but also the attenuation modulus is found to decrease in a regular manner, as the organic modifier concentration is increased in the eluent; this can be interpreted as evidence of a competition between IIR, methanol, and analyte for the inner layer sites. When the neutral analyte is not expected to be able to penetrate it, that is when it is not an amphiphylic surface active compound, only retention and not

Table 1

Influence of Methanol Concentration in the Buffered Mobile Phase on Retention Modulus*

	Methanol Concentration in the Eluent (v/v)			
	10%	**15%**	**20%**	**25%**
η 25 TMU	1.522	1.457	1.386	1.225
η 25 DMF	1.232	1.158	1.104	1.048
η 25 DMC	1.078	1.065	1.057	1.022

* Conditions: Column: 15 cm x 4.6 mm I.D. containing 9 µm Polyspher C_{18}, (C_{18}-SDVB), Merck. Injection volumes: 0.1µL of DMF 9.4 g/L, 2 µL of DMC 7.9 g/l and 1 µl of TMU 9.7 g/l. Flow-rate: 0.700 mL/min. Eluent: 81.6 mM phosphate buffer pH 7.2 - methanol, (see table for percentages v/v) containing tetrabutylammonium phosphate 0-25 mM. For explanations of η_{25} values see text.

Table 2

Influence of Methanol Concentration in the Aqueous Mobile Phase on Retention Modulus*

	Methanol Concentration in the Eluent (v/v)			
	10%	**15%**	**20%**	**25%**
η 25 TMU	1.411	1.230	1.201	1.156
η 25 DMF	1.085	1.056	1.043	1.022
η 25 DMC	1.042	1.018	1.007	1.002

* Conditions: Column: 15 cm x 4.6 mm I.D containing 9 µm Polyspher C_{18}, (C_{18}-SDVB), Merck. Injection volumes: 0.1µL of DMF 9.4 g/L, 2 µL of DMC 7.9 g/L and 1 µL of TMU 9.7 g/L. Flow-rate: 0.700 mL/min. Eluent: water - methanol, (see table for percentages v/v) containing tetrabutylammonium phosphate 0-25 mM. For explanations of η 25 values see text.

the retention modulus is influenced by methanol concentration in the eluent. The presence of large percentages of methanol in the eluent may minimize or finally mask the decrease of the retention with increasing IIR concentration, since it “buffers” the competition between tested analytes and lipophilic ions.

This, together with the analyte nature (see below), can provide the rationale for the possible lack of neutral molecules retention dependence on IIR concentration in the mobile phase, which was postulated[4-5,18] and experimentally confirmed.[4,18-21]

From a comparison of results shown in Table 1 and 2, it can be underlined that η 25 values obtained for the buffered mobile phase are higher than those obtained with the aqueous eluent if the methanol concentration is the same. This can be easily explained since the buffer provides those counter ions which are able to reduce the charge repulsion thereby increasing the amount of adsorbed lipophilic reagent.[22]

We now want to discuss the influence of ionic strength on neutral molecules retention in Ion Interaction chromatography. It has been shown[16] that for a neutral molecule ln k increases linearly with increasing salt concentration, since salt is able to increase the eluent surface tension, (γ), according to the following equation:

$$\gamma = \gamma_0 + \tau\, m \qquad (15)$$

where γ_0 is the surface tension of pure water, τ is a coefficient which depends on the salt nature, and m is the solution molality.

On the other hand, according to the Stern-Gouy-Chapman theory of double layer adsorption[23-25] the surface concentration of IIR increase with increasing ionic strength. Hence with increasing eluent ionic strengths of both the neutral molecules retention and IIR retention should increase.

As may be seen from Table 3 and Table 4, neutral molecule retention decreases with increasing ionic strength, whereas IIR concentration in the eluent is constant. Since this is the opposite of what is expected, on the basis of solvophobic theory, if IIR is not present in the mobile phase, it is clear that neutral molecule retention decreases, since the amount of reagent adsorbed increases with increasing ionic strength even if its concentration in the eluent is constant: again, when the eluent salt concentration is higher, the charge repulsion at the inner layer on the stationary phase is reduced, and the amount of adsorbed lipophilic reagent increase.[22]

It can be concluded that the effect of the increased retention subsequent to the increased ionic strength is higher for IIR than for test neutral compounds. However, it can not be a priori excluded that for more lipophilic test compound data the opposite can be observed.

Table 3

Influence of Eluent Ionic Strength on Retention at Low IIR Concentration*

	Phosphate Concentration (mM)		
	0.0	**40.8**	**81.6**
k TMU	4.750	4.126	4.063
k DMF	1.103	1.033	1.021
k DMC	1.970	1.960	1.852
ionic strength	0.0150	0.0966	0.1782

* Conditions: Column: 15 cm x 4.6 mm I.D. containing 9 µm Polyspher C_{18}, (C_{18}-SDVB), Merck. Injections volumes: 0.1µL of DMF 9.4 /L, 2 µL of DMC 7.9 g/L and 1 µL of TMU 9.7 g/L. Flow-rate: 0.700 mL/min. Eluent: phosphate buffer pH 7.2 (see table for variable phosphate concentration) - methanol, 85:15 (v/v) containing tetra butylammonium phosphate 10 mM.

Table 4

Influence of Eluent Ionic Strength on Retention at High IIR Concentration*

	Phosphate Concentration (mM)		
	0.0	**40.8**	**81.6**
k TMU	4.385	3.7778	3.768
k DMF	1.096	0.9875	0.979
k DMC	1.914	1.900	1.839
ionic strength	0.0376	0.1191	0.2007

* Conditions: Column: 15 cm x 4.6 mm I.D. containing 9 µm Polyspher C_{18}, (C_{18}-SDVB), Merck. Injections volumes: 0.1µL of DMF 9.4 g/L, 2 µL of DMC 7.9 g/L and 1 µL of TMU 9.7 g/L. Flow-rate: 0.700 mL/min. Eluent: phosphate buffer pH 7.2 (see table for variable phosphate concentration) - methanol, 85:15 (v/v) containing tetra-butylammonium phosphate 25 mM.

From an accurate analysis of data shown in Table 3 and 4, it can be confirmed[1] that if the phosphate concentration is the same, retention is lower when the IIR concentration in the eluent is higher; at variance with Bidlingmeyer's ion-interaction mechanistic model postulate for neutral

molecules.[4-5] The findings reported here, underline that it can not be taken for granted that the free energy of the adsorption of a charged species is independent on the potential difference between the surface of the stationary phase and the mobile phase. By taking this into account, the electrostatic theory for ion-interaction chromatography of oppositely charged analytes and IIR will be further improved.

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