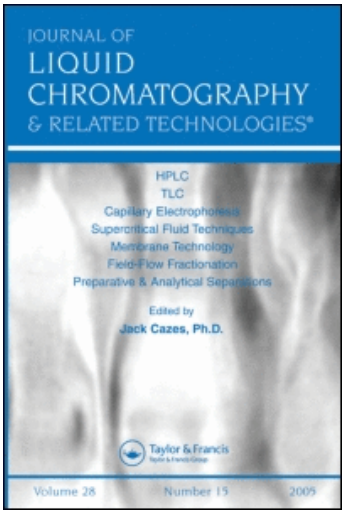

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RETENTION BEHAVIOR OF NEUTRAL MOLECULES IN ION INTERACTION CHROMATOGRAPHY

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ABSTRACT

The retention behaviour of aprotic neutral molecules in Ion Interaction Chromatography was investigated. The study demonstrates that their retention, for an aqueous mobile phase (phosphate buffer pH 7.2-methanol, 85:15 v/v) containing the ion-interaction reagent (IIR) in the range 0-25 mM, decreases with increasing IIR concentration. Silanophilic interactions were accounted for by comparison of results obtained with either a silica based or a styrene-divinylbenzene based C_{18} stationary phase, under otherwise identical conditions. The retention behaviour of neutral molecules was elucidated on the basis of the exhaustive Ion Interaction retention model and evidence was obtained to support the proposition that the decreased retention is the result of an adsorption competition between tested analytes and ion-interaction reagent for inner layer sites on the stationary phase.

INTRODUCTION

The use of reverse phase ion-interaction chromatography has become a widely used separation mode in analytical LC[1] because it provides a useful alternative to ion exchange chromatography.[2]

According to the retention model of Bidlingmeyer,[3-4] which is broader in scope than either a dynamic ion-exchange mechanism[5-7] or an ion-pair mechanism[8-9] the lipophilic IIR, flowing under isocratic conditions, dynamically adsorbs onto the alkyl-bonded apolar surface of the stationary phase, forming a primary charged ion layer. The corresponding counter ions are found in the diffuse outer region to form an electrical double layer. The retention of the sample involves its transfer through the electrical double layer, hence it results from both electrical and van der Waals forces by means of a mixed retention mechanism.

While the effect of ion interaction reagents on the retention factor (k) values of oppositely charged analytes has been well studied,[1-4,10-12] the influence of ion-interaction reagents on the k values of neutral species has not been widely investigated. For uncharged analytes no retention dependence on ion-interaction reagent concentration in the eluent was postulated.[3-4] This hypothesis was experimentally confirmed.[3,13-14] Even if Kong et al.[15] interpreted their data as indicating that the retention of their analytes (weak acids) was essentially independent of IIR concentration at pH 3.6 a residual increase of retention can be found from the analysis of their data. This is not unexpected since the ionogenic analytes, in the ionized form may interact with the positively charged IIR thereby providing the observed slight increase in retention.

However, even if it is generally accepted[1] that neutral analytes retention does not depend on IIR concentration, some examples which point to the contrary are present in the literature.[11-12,16-18] In the work of Knox and Hartwik[16] the authors noticed a decrease of benzyl alcohol and phenol retention with increasing respectively octyl sulphate and tetrabutylammonium (TBA) concentration in the mobile phase buffered at pH 6.0. Since the tested molecules are good hydrogen bond donors, silanophilic interaction could not be deconvolved from the effect of the IIR presence in the eluent; moreover TBA, at the very low concentration at which it was used, reacts nearly exclusively with the active silanol groups.[1,19] Hence, it can not be taken for granted that the decrease in retention was the result of the adsorption of the IIR at the stationary phase surface. Melin et al.[11] developed a retention model for neutral molecules based on the limited classical ion-pair theory, but they had to conclude that retention was a function of the IIR concentration and not for the full range of neutral compounds they studied. Hung and Taylor[12] proposed a mechanism of retention for neutral molecules based on dynamic ion exchange chromatography, but their experimental results were at variance with that predicted by the theory

they developed. However, no rationalization which takes into account that the silanol group is a very strong adsorption site[20] and that a very large percentage of the n-alkylammonium ions are electrostatically interacting with surface silanols,[19] has been produced.

The overall impression gained from these literature reports was that there is a wide variability among experimental results and the generally accepted lack of neutral molecules retention dependence on IIR concentration is more an assumption than a general experimental evidence. The reason for the literature experimental results variability has never been elucidated. The range of compounds which were tested was not intentionally designed to cover the chemical and physical properties which are supposed to be a prerequisite, on the basis of the Ion Interaction Model premises, for the retention decrease with increasing IIR concentration.

Furthermore, the selected experimental conditions were not designed to deconvolve silanophilic from dispersion interactions, to seek their relative importance, and to pinpoint the exact source of the observed phenomenon, hence its exact origin was not clear. Moreover, to our knowledge,[1] no mechanisms, which takes into account the exhaustive Ion Interaction retention model findings, were developed to understand the retention behaviour of neutral molecules. Hence, there remains a strong need to urge that this very flexible separation technique not be viewed as being a completely understood one.

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[2] for inner layer sites on the stationary phase. Taking this into account, we would expect to find a decrease of the capacity factor of this kind of neutral analytes with increasing ion-interaction reagent concentration.

This hypotheses is at variance with the generally accepted rationalization[1] of the influence of IIR concentration in the mobile phase on neutral molecules retention, hence we designed some experiments to test it. Moreover, we wanted to seek evidence to support the proposition that uncharged molecules do experience a change in retention in presence of IIR when compared to eluents containing no reagent as modifier.

Since, to date, no attempt to deconvolute silanophilic from reversed phase interactions were made, we compared the results obtained with a silica based C_{18} stationary phase with those relative to a C_{18} functionalized styrene-divinylbenzene (SDVB) chromatographic bed equilibrated with the same mobile phases.

An explanation for the discrepancies between our results and the current rationalization of Ion Interaction Chromatography[3-4] of neutral molecules is attempted.

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 230 nm for detection of dimethylformamide (DMF), dimethyl sulfoxide (DMSO). Analytes injection volume was 0.1μL for DMF and DMC and 0.4 μL for DMSO. The column was thermostated at 28°C.

All experiments were carried out with the following commercial stainless steel column: a) 25 cm x 4.6 mm I.D., packed with 5 μm Res Elut 5 C_{18}, for reverse phase chromatography, purchased from Varian (Walnut Creek, CA, USA); b) 15 cm x 4.6 mm I.D containing 9 μm Polyspher C_{18} (C_{18} functionalized SDVB copolymers particles), purchased from Merck.

The eluent flow-rate was 0.500 mL/min with the latter column and 0.833 mL/min with the former.

We decided to maintain, in the ODS stationary phase, the same eluent velocity which was present in the C_{18} functionalized SDVB chromatographic bed, in order to reproduce the same conditions and hence, to allow a consistent comparison of the experimental results. The right flow rate in the first column was calculated on the basis of the equation between total void volumes ratio and flow rates ratio in the two systems.

The hold-up time (t_0) was determined by injecting 25 μL of water and measuring the time from injection to the first deviation from the baseline. Tetrabutylammonium bromide, dimethylformamide, dimethyl sulfoxide, and acetone 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 consisted of 81.6 mM phosphate buffer pH 7.2 - methanol, 85:15 v/v containing tetrabutylammonium bromide in the concentration range from 0 to 25 mM. All solutions were filtered through a 0.45 μm pore size regenerated cellulose filter (Schleicher&Schuell, Dassel, Germany). 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 0.500 mL/min and 0.833 mL/min respectively. Equilibration was established by obtaining similar results in duplicate runs at a 15 min interval.

RESULTS AND DISCUSSION

In this report, we present and discuss the results obtained in the study of the retention behaviour of neutral, non ionogenic molecules in ion interaction chromatography

A test for neutral analytes, it was planned to select non ionogenic, aprotic molecules. The number of analytes we tested was limited by the UV transparency of most of the small, polar, surface active molecules which are supposed to be able to penetrate the inner region of the double layer by direct competition with the IIR.[2] The counter ion of TBA was chosen to be the bromide ion in order to minimize the mobile phase pH variation upon ion-interaction addition.

Adsorption interactions of neutral polar analytes with the "uncovered" C_{18} surface and hence, competing equilibria with ion-interaction reagent, should prevail at low organic modifier concentration. In order to shed light on and magnify the effect of this competition, we therefore, decided to sharpen the reverse phase operating conditions by studying their retention behaviour with a mobile phase containing the minimum percentage of added organic modifier. The resistance of the SDVB stationary phase to "wetting" by aqueous media is so strong that use of eluents containing more than 90% water is advised against because they can cause void formation, hence, to be safe, 15% methanol was used in the mobile phase of both columns in order to make the results comparable. In a separate experiment with the silica based stationary phase, 100% buffer was used as eluent in order to give evidence to the influence of methanol concentration in the mobile phase on analyte-IIR competing equilibria.

We extended the range of IIR concentration up to 25 mM in order to reach the concentration at which it is able to induce a stable modification of the stationary bonded phase surface.[1]

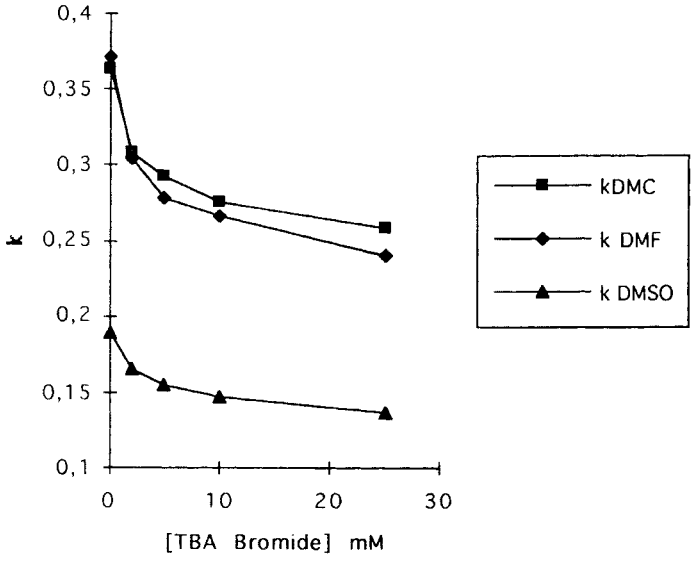

Figure 1. Effect of IIR concentration upon the retention of neutral analytes on a silica based stationary phase. Conditions: Column: 25 cm x 4.6 mm I.D. 5 mm Res Elut 5 C_{18} (Varian). Injections of 0.1mL for DMF and DMC and 0.4 mL for DMSO; eluent flow-rate: 0.833 mL/min. Eluent: 81.6 mM phosphate buffer pH 7.2 - methanol, 85:15 v/v containing tetrabutylammonium bromide in the concentration range from 0 to 25 mM.

The attenuation in retention caused by IIR presence in the eluent is conveniently expressed by the modulus, η, that is defined by:

$$\eta = k_0 / k$$

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 parameters 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 Ion Interaction Chromatography of neutral molecules.

Figure 1 shows the effect of IIR concentration, in the range 0-25 mM, on the capacity factor of uncharged solutes chromatographed on the silica based C_{18} stationary phase. In each case k decreases rapidly between 0 and 5 mM TBA, and then remains relatively constant with further increases in ion-interaction reagent concentration.

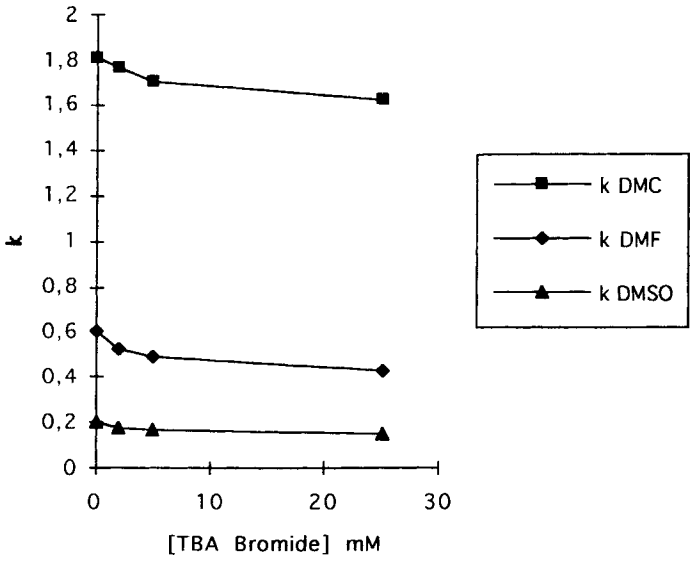

Figure 2. Effect of IIR concentration upon the retention of neutral analytes on a SDVB based stationary phase. Conditions: Column: 15 cm x 4.6 mm I.D containing 9 mm Polyspher C_{18}, (Merck). Injections of 0.1mL for DMF and DMC and 0.4 mL for DMSO; eluent flow-rate: 0.500 mL/min. Eluent: 81.6 mM phosphate buffer pH 7.2 - methanol, 85:15 v/v containing tetrabutylammonium bromide in the concentration range from 0 to 25 mM.

This pattern is not qualitatively different from that observed when the charge status of the analyte and the IIR is the same[3-4,11] even if in this instance there are also ion exclusion phenomena which stress the retention dependence on IIR concentration. As shown in Figure 1, with increasing TBA concentration, the analytes are eluted according to their polarity, the retention order being caused basically by lipophilic and not electrostatic forces. An inversion can be observed for DMF and DMC if no added IIR is present in the mobile phase: this can be explained by taking into account silanophilic interactions as confirmed by the elution order which was obtained on the Polyspher column with the same mobile phase. Figure 2 features the course of the retention factors, for the same analytes, obtained with the Polyspher column under otherwise identical conditions. The point of interest is that, even if silanophilic interactions are not present, the same kind of dependence can be obtained, thereby demonstrating that adsorption competition between polar surface active molecules and IIR do take place also on the hydrophobic stationary phase; this competition has to be expected since both the tested neutral molecules and TBA ions are amphiphilic compounds.

Table 1

Influence of Experimental Conditions on Retention Modules*

	$\eta 25C_{18}$-SDVB Buffer: MeOH 85:15 v/v	$\eta 25C_{18}$-silica Buffer: MeOH 85:15 v/v	$\eta 25C_{18}$-silica Buffer: MeOH 100:0 v/v
DMC	1.11	1.40	1.68
DMF	1.39	1.54	1.92
DMSO	1.39	1.39	1.62

*Conditions: Columns a) 25 cm x 4.6 mm I.D. 5 μm Res Elut 5 C_{18}, Varian (C_{18}-silica), b) 15 cm x 4.6 mm I.D containing 9 μm Polyspher C_{18}, (C_{18}-SDVB), Merck. Injections of 0.1 μL for DMF and DMC and 0.4 μL for DMSO; eluent flow-rate: 0.500 mL/min with the latter column and 0.833 mL/min with the former. Eluent: 81.6 mM phosphate buffer pH 7.2 - methanol, (see table for percentages v/v) containing tetrabutylammonium bromide 0 - 25 mM. For explanation of η_{25} see text.

As it may be seen from Table 1, in the Polyspher column, more polar neutral molecules (as DMSO, DMF) are characterized by retention moduli larger than that of less polar analytes (as DMC). This was interpreted as indicating that the competition of the former with lipophilic ions is stronger: since the common driving force for competing adsorption is the reduction in the high surface tension of the stationary phase,[2] the higher the analyte surface activity is, the more it is expected to compete with IIR for inner layer of the stationary phase. When residual silanols are present in the chromatographic bed, the modulus values do not show such a dependence on the molecular polarity thereby indicating that silanophilic interactions are operating. Another point of interest shown in Table 1 is that higher, or at least similar (for DMSO), retention modulus values were obtained with the silica based stationary phase if compared to those measured in the C_{18}-SDVB chromatographic bed under otherwise identical conditions. Again, this is indicative of the strong electrostatic interaction of TBA ions with the negative charges of the dissociated residual silanols, their pKa being 4.5.[21]

The experimental results support the fact that the tested neutral molecules, at variance with the Bidlingmayer ion-interaction mechanistic model, do experience a change in retention in the presence of IIR when compared to eluents containing no reagent as modifier. We would also like to underline that

neither the ion-pair nor the ion-exchange models can give a clear rationale for the competition between tested analytes and IIR for inner layer sites on the stationary phase.

To explain why our results underscore a rationalization of the influence of IIR concentration in the mobile phase on neutral molecules retention, different from the generally accepted one,[1] two points must be to be taken into account.

First, the phenomenon we have described is particularly expected to occur when low organic modifier is present in the aqueous mobile phase. It is well known that an increase in its concentration in the eluent will result in reduced retention (and hence reduced interactions with the stationary phase) of the sample[1] via competition with the ion-interaction reagent.[2] But, the presence of organic modifier is expected to also decrease retention modulus of surface active neutral molecules, since it acts as a second competitor of IIR, hence it "buffers" the competition between tested analytes and lipophilic ions.

As demonstrated in Table 1, the modulus values for the silica based reversed stationary phase, with no added organic modifier in the mobile phase, are higher than those obtained under otherwise identical conditions when the eluent contained 15% methanol. Thus, organic modifier presence in the eluent may minimize or eventually mask the decrease of the retention with increasing IIR concentration, thereby providing the rationale for the possible lack of neutral molecules retention dependence on IIR concentration in the mobile phase.

Second, the nature of the analyte molecule can influence the dependence of k on the lipophilic ion concentration: as demonstrated, in absence of superimposed silanophilic interactions, the more the analyte surface activity, the stronger this dependence will be. The contemporary presence of hydrophobic and hydrophilic moieties in the tested analyte, which give them surface active properties, led to the expected results.

While evidence was obtained to support the existence of competing equilibria between tested neutral analytes and lipophilic ions and their influence on analyte retention, there remains a need for a systematic study of this phenomenon to *a priori* estimate, on the basis of simple molecular characteristics, the tendency of an analyte to compete with IIR.

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