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Demonstration of simultaneous anion-exchange and reversed-phase behavior on a strong anion-exchange column

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Abstract

We demonstrate in this report that a conventional silica-based strong anion-exchange column can exhibit reversed-phase chromatographic behavior simultaneously with ion-exchange in a methanol–aqueous phthalate mobile phase. Reversed-phase behavior is shown for PAHs in relatively high methanol-content mobile phases, while polar organics exhibit reversed-phase behavior in 0–10% (v/v) methanol–water eluents. At low concentrations (0–10% v/v) methanol has little or no effect on anion retention, while the anions exhibit only slight increases in retention in 60–80% (v/v) methanol at near-neutral pH values. Likewise, pH changes used to manipulate anion retention have essentially no effect on PAH retention, and cause only small decreases in retention for most of the polar organics studied. Little or no pH-effect was seen on a phenyl column (no exchange group) or with acetate mobile phase. It is shown that manipulation of pH and methanol content allows the grouping of neutral organic analytes early in a chromatogram, followed by the anions, with no class overlap. Several example chromatograms are given, including that of a red wine sample. Column efficiency was good for anions but only modest for organics.

Keywords: Retention mechanisms; Stationary phases, LC; Mobile phase composition; Anion-exchange; Anions

1. Introduction

For several years our laboratory and others have been interested in developing liquid chromatographic methods for the simultaneous separation of analytes of different classes. In particular, the ability to separate and quantitate both ionic and neutral species in one run would reduce both cost and analysis time. We have employed ion-interaction chromatography [1], HPLC phases synthesized in our laboratory [2], as well as commercially available phases for the separation of inorganic anion– [3] and metal cation–neutral organic mixtures [4]. The latter two reports also describe work by other laboratories.

It has been known for 30 years or more that organic polymer-based ion-exchange resins could be used for extraction [5] and chromatographic separation [6,7] of neutral organic analytes. Recent work also demonstrated that both reversed-phase and ion-exchange mechanisms operate simultaneously and essentially independently on silica-based, strong-acid cation-exchange HPLC phases [4]. The organic portion of the appendages and end-capping moieties afford reversed-phase character and the attached sulfonate ionophore, of course, provides ion-exchange ability.

That similar behavior would obtain on strong anion-exchange phases is implied by several earlier studies from this laboratory and that of others. Work employing serial columns [8] or a mixed-bed of reversed-phase and anion-exchange materials [9] has been successful in separating mixtures of anions and uncharged analytes in one run. The reversed-phase and anion-exchange mechanisms were shown to operate independently of each other in the mixed-bed column [9]. Mixed ligand (C_8 and strong anion-exchange appendages) [10] and multifunctional phases (different interaction sites on one attached ligand) [11] were also employed for both anions and neutral species. Commercial multifunctional phases containing an embedded weak anion exchanger and a C_8 or C_4 group were also used for inorganic anions and weak organic acids [12]. Haddad and Croft ascribed the separation of a mixture of organic and inorganic anions on a styrenedivinylbenzene-based

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anion-exchange column [13] to both ion-exchange and reversed-phase interactions. Heinitz et al. have discussed the use of multifunctional or mixed-mode phases in the context of protein separations and found that both ionic and hydrophobic interactions occurred [14].

We have used phases synthesized in our laboratory as well as the commercial C_8/anion-exchange phase mentioned above [12] to simultaneously separate both anions and neutral organic analytes [3]. Further, a phase containing metal-complexing, weak anion-exchange, and reversed-phase portions was able to separate a mixture of metal cations, inorganic anions and neutral organics isocratically in one run [2].

As seen above, most of the mixed-mode work so far has employed weak anion-exchange phases. But silica-based strong anion-exchangers are more widely employed in chromatographic laboratories, and are available off-the-shelf. As many environmental, pharmaceutical and food samples require that both anions and neutral organics be determined, it was of interest to show that both reversed-phase and anion-exchange mechanisms are exhibited and operate essentially independently on these phases, as was true for the strong cation-exchange phases [4]. We also wanted to demonstrate that simultaneous separation and detection of mixture of anions and neutral organics could be efficiently performed. It should be noted that as ion-exchange phases have charged sites that are highly hydrated, they are compatible with high water content mobile phases, unlike some conventional reversed-phase columns. And so it was of interest to show that some anion–neutral organic separations could be done in 100% aqueous mobile phases as was done on a strong cation-exchange column [4]. Lastly, while mixed-bed and designer mixed-ligand phases offer the ability to alter the ratio of ion-exchange-to-reversed-phase sites [9,10] most researchers will choose readily available commercial phases, and so we have employed such columns in this report.

2. Experimental

2.1. Apparatus

The chromatographic system consisted of a Kratos Spectroflow 400 dual-piston pump; a Rheodyne Model 7010 injection valve with a 10 μl sample loop; and an ISCO V^4 variable-wavelength ultraviolet-visible detector. UV detection of mixtures of small organic compounds and anions (indirect detection) [15] was performed at 260 nm, while that of mixtures of PAHs and anions was usually done at about 300 nm. Chromatographic data was acquired with the EZChrom Chromatography Data System, version 6.7, from Scientific Software Inc. A Fisher Accumet 915 pH meter was used to acquire pH data.

The analytical column was 150 × 4.6 mm, packed with spherical 5 μm, 100 Å pore Nucleosil quaternary ammonium strong anion exchanger (Machery-Nagel, from Alltech Associates). The stationary phase was trimethylsilyl end capped, making it a mixed-ligand, multifunctional material. Two of these columns were used during the course of this work. A guard column packed with 12 μm, 300 Å, spherical strong anion-exchange phase was used in later phases of the investigation. A Zorbax phenyl column (250 × 4.6 mm) was used as a comparison with the anion-exchange column in the pH-effect part of this study.

2.2. Reagents and solutions

All mobile phases and analyte solutions were made up in water purified with a Nanopure (II) system (Barnstead). Reagents were either HPLC or ACS grade. Mobile phase solvents (water and methanol), mixed mobile phases and analyte solutions were all filtered through 0.45 μm membrane filters. Anion stock solutions were made up in 100% water. Organic analyte stock solutions were prepared in 100% methanol or some mix of methanol and acetone for the larger PAHs. The pH of mobile phases was adjusted with either 2 M nitric acid or 2 M sodium hydroxide. Stock solutions of anions and organics were diluted in the appropriate mobile phase to prepare samples for injection. Injected analyte concentrations were typically 10^{-2} or 10^{-3} M for anions, 10^{-3} or 10^{-4} M for smaller organics, and as low as 10^{-7} M for crysene.

The pH meter was calibrated with aqueous buffers. It should be noted that mobile phases containing methanol yielded values more basic than would be the case in an all-water mobile phase, but that the measured pH values are very close to actual hydrogen ion activity [16].

2.3. Chromatographic procedures

All chromatography was performed at ambient temperatures. The flow rate was usually 1.0 ml min^{-1}. Operating pressures were in the range of 1500–2000 psi. Usually the methanol content of the injected sample was different from that of the mobile phase, and so a distinct injection peak resulted which was used to obtain the column dead time (t_m). Although the shape of the injection peak(s) differed from one mobile phase to another, especially if the pH were changed, it was found that the position of the maximum positive deflection from the base line remained virtually constant. The time at which this occurred was taken as t_m. The column was flushed for 30 min once or twice a week with an appropriate mixture of methanol and water to remove salts. Then methanol was run through the guard column for 30 min and the analytical column for 30 min.

Indirect UV detection of anions was accomplished using potassium hydrogen phthalate (KHP) eluting agent [15]. At 260 nm, where the small, polar organics were detected, 2 mM KHP was used. At 300 nm, used for PAH detection, the hydrogen phthalate anion absorbs only weakly [3] so that 5 mM KHP eluent was needed.

3. Results and discussion

The strong anion-exchange phase used in this study is a conventional benzyl-trimethyl ammonium phase linked to the silica surface by a propyl chain. As this is a quaternary ammonium material, the anion-exchange site is always charged and so its contribution to anion retention should be pH independent. The propyl chain and the phenyl moiety afford some dispersion interaction sites to the column, as do the trimethylsilyl end capping groups, essentially a C_1 phase [4]. And so this mixed-ligand, multifunctional material might be expected to operate in both the anion-exchange and reversed-phase modes. This is demonstrated below.

3.1. Mobile phase effects

In order to separate anions from one another by *k* regulation, the pH and/or ionic strength of the mobile phase must be manipulated. To adjust *k* for neutral organic analytes the organic cosolvent concentration of the mobile phase is changed. The present work used methanol as the cosolvent and potassium hydrogen phthalate (KHP) as the anion eluting agent. The KHP, as shown below, also permits indirect detection of anions that do not absorb in the UV-Vis region [3,15]. We present here studies of how pH manipulation affects the retention of neutral organic analytes, and how anions respond to methanol cosolvent changes. Work was performed in two methanol concentration ranges. Small polar organic analytes were determined in the 0–10% (v/v) methanol–aqueous region; white PAH work was done in the 60–80% (v/v) methanol–aqueous range. The organic analytes were mixed with anions in both methanol ranges.

3.1.1. Typical chromatograms

In Fig. 1 are shown typical chromatograms of mixtures of neutral organics and anions, in both high- and low-methanol mobile phases. Earlier reports from this laboratory noted that neutral organic analytes could be eluted from commercial cation-exchange columns at relatively low organic cosolvent concentrations [3,4]. Similar behavior was found in the present study on the strong anion-exchange phase. In Fig. 1 (top) for example, it can be seen that pyrene, a four-ring PAH, is eluted with a *k* of about 2.6 with only 60% (v/v) methanol. In Fig. 1 (bottom) *p*-nitroaniline is seen to elute with a *k* of 7.7 in 3% methanol. In a 100% aqueous mobile phase *p*-nitroaniline has a *k* of about 11 (not shown).

The anions in these mixed ionic-neutral samples are seen to have negative peaks, a consequence of the indirect detection mode employed [4,15]. The KHP eluting agent provides a positive background absorbance at the wavelength used, so that when a hydrogen phthalate ion replaces and elutes an analyte ion the reduced absorbence yields a negative peak. This result lends some convenience in visualizing the anions

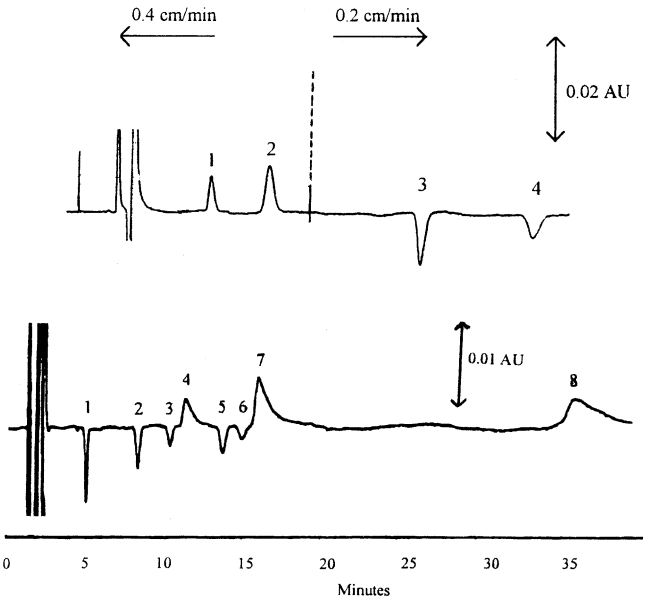

Fig. 1. Simultaneous determination of inorganic anions and neutral organics on a strong anion-exchange column. Top: mobile phase was methanol–water (60:40, v/v), 5 mM potassium hydrogen phthalate, pH 6.6: (1) phenanthrene, (2) pyrene, (3) chloride, (4) nitrate. Detection: UV at 301 nm. In order to compress the "*x*" axis, two different chart speeds were used, as shown on either side of the dashed vertical line. Bottom: mobile phase was methanol–water (3:97, v/v), 2 mM potassium hydrogen phthalate, pH 5.5: (1) iodate, (2) chloride, (3) nitrite, (4) *p*-chloroaniline, (5) bromide, (6) nitrate, (7) *p*-nitroaniline, (8) *p*-nitrophenol. Detection: UV at 260 nm.

in the presence of the positive peaks of neutral analytes, and in providing similar sensitivity for all like-charged, nonabsorbing anions.

3.1.2. Effect of methanol on neutral organic retention

Given that the strong anion-exchange phase can retain and separate neutral organic molecules the question remains as to whether this behavior is similar to that of typical reversed-phase columns. Fig. 2A shows the retention behavior of two PAH analytes from 60 to 80% (v/v) methanol in the 5 mM KHP mobile phase used to elute anions in this methanol range. Fig. 2B depicts the behavior of some polar organic analytes from 0 to 10% (v/v) methanol in 2 mM KHP. In both cases a *k* decrease of about 2–2.5 is found with a 10% (v/v) methanol increase, typical reversed-phase behavior [17–19]. It would appear then that reversed-phase behavior is operative on strong anion-exchange phases even in the presence of mobile phase conditions that allow anion-exchange. Note too that the polar organic analytes were run in 100% aqueous conditions (Fig. 2B) and that little or no discontinuity is found in the *k* versus percent methanol plot indicating continued wetting of the stationary phase within the pores and no folding or collapse [20,21]. The quaternary ammonium ionophore apparently keeps the appendage end solvated and extended into the mobile phase within the pores even in the absence of methanol. In this regard the ion-exchange phases appear to act like

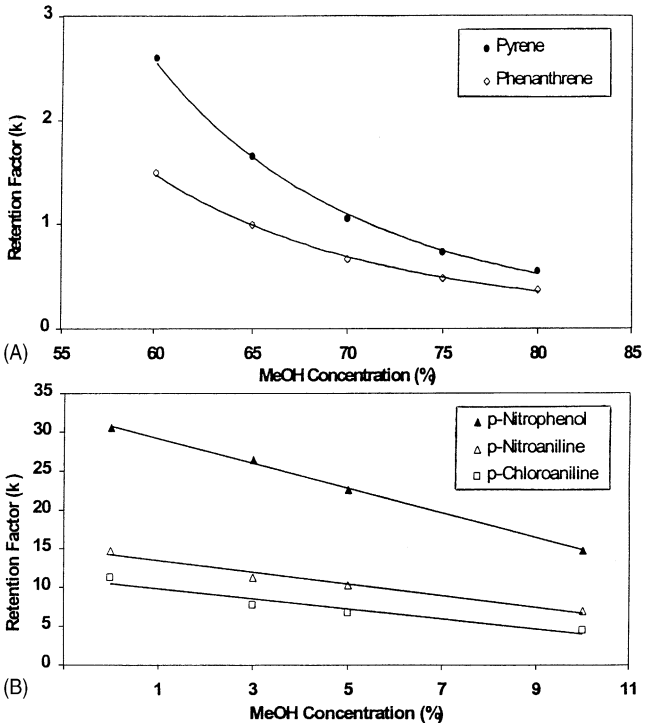

Fig. 2. Effect of methanol on the retention factor of neutral organic analytes on a strong anion-exchange column. Mobile phase: (A) 5 mM potassium hydrogen phthalate, pH 6.7 and (B) 2 mM potassium hydrogen phthalate, pH 5.5.

the polar-embedded phases that have become so popular of late [22].

3.1.3. Effect of methanol on anion retention

Again data was obtained at both high and low mobile phase methanol content. Fig. 3A shows the retention behavior of chloride and nitrate from 60 to 80% (v/v) methanol in 5 mM KHP, while Fig. 3B reports *k* values for five anions from 0 to 10% (v/v) methanol in 2 mM KHP. In the high-methanol mobile phases chloride coeluted with nitrite and bromide coeluted with nitrate. At low methanol content good separation was possible for all five anions (Figs. 1 (bottom) and 3B). In both methanol ranges studied *k* increased with increasing methanol concentration, in contract to small decreases in cation *k* values found on a strong cation-exchange phase [4]. The *k* increases were small at low methanol content, about 0.1–0.6 with a 10% (v/v) increase in methanol. At higher methanol content (and higher KHP concentration) *k* increases were from 0.5 to 1.5 with a 10% (v/v) methanol increase.

The literature is somewhat inconclusive concerning the effect of organic cosolvent on anion retention. Organic anions display some reversed-phase behavior, especially on organic polymer resin substrates, and exhibit a *k* decrease at low organic content [23]. At higher organic content, above 30% (v/v), *k* increases are found [13,24] for both organic and inorganic anions with an increase in organic cosolvent, as the present study found.

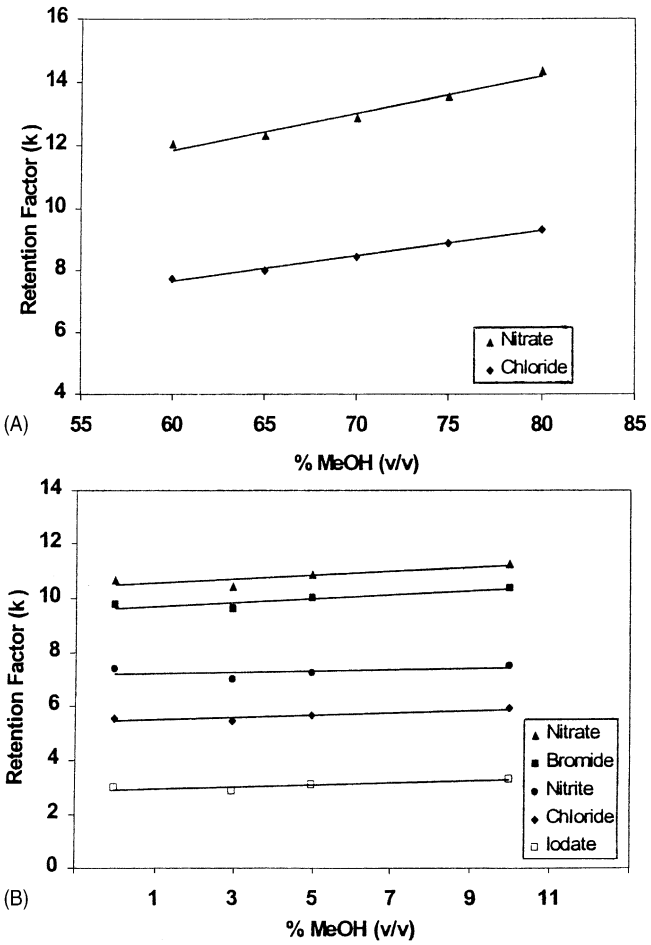

Fig. 3. Effect of methanol on the retention factor of inorganic anions on a strong anion-exchange column. Mobile phase: (A) 5 mM potassium hydrogen phthalate, pH 6.7 and (B) 2 mM potassium hydrogen phthalate, pH 5.5.

Not much literature data is available for inorganic anions at low organic cosolvent content, however. One study found a decrease in *k* for phosphate in the 0–20% (v/v) acetonitrile range [13], but most studies looked only at the high-organic ranges. In the present study, although an overall *k* increase was found on going from 0 to 10% (v/v) methanol, all five anions studied showed a very slight *k* decrease (about 0.1–0.3) on going from 0 to 3% methanol (v/v), followed by a small *k* increase. We are aware of no literature corroboration of this finding. It should be noted here that each data point is the result of three sets of three injections each, randomly distributed over a 2–3 weeks span with mobile phase made from scratch for each of the three series of runs. The standard deviations in *k* at 3% (v/v) methanol range from 0.02 to 0.06, well below the *k* decreases found on going from 0 to 3% methanol.

In general, though, *k* increases with increasing methanol content for these inorganic anions. At least two effects are operative here. First, a decrease in dielectric constant of the mobile phase with increasing methanol results in a lessening of the solvation of the inorganic anions and stronger

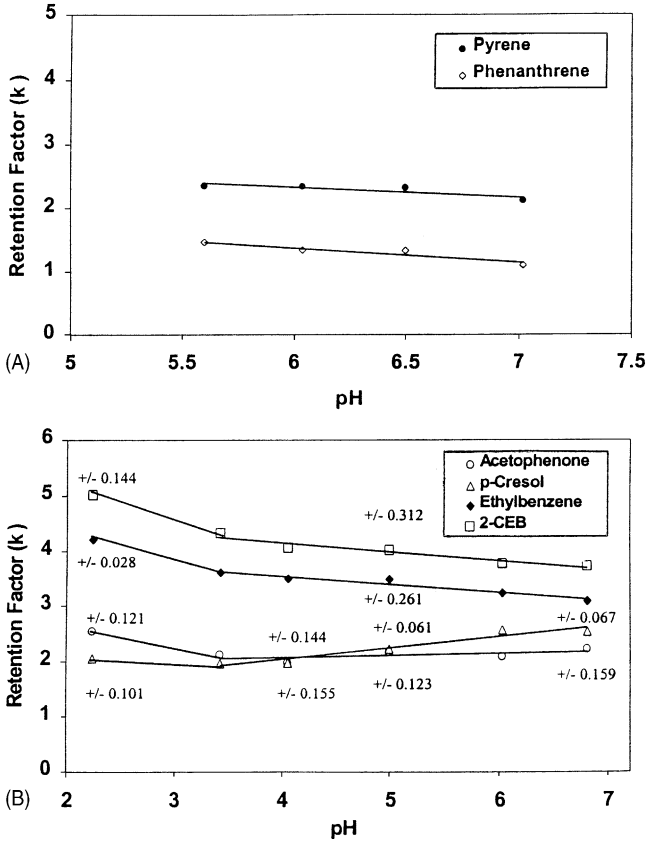

Fig. 4. Effect of pH on the retention factor of neutral organic compounds on a strong anion-exchange column. Mobile phase: (A) methanol–water (60:40, v/v), 5 mM potassium hydrogen phthalate and (B) methanol–water (10:90, v/v), 2 mM potassium hydrogen phthalate. CEB is 2-chloroethylbenzene.

interaction with the stationary phase ammonium site [24]. Second, the ionization of the phthalic acid is repressed by the methanol, making the KHP a weaker eluent as methanol is added [13,16]. Still, ion-exchange behavior in mixed solvents is complicated, and the major contributors to retention change are difficult to determine.

3.1.4. Effect of pH on retention of organic neutrals

The effect of pH changes on PAH retention was examined over a narrow range in a methanol–water (60:40), 5 mM KHP mobile phase, and is shown in Fig. 4A. Only minimal changes in *k*, a slight decrease, are seen for pyrene and phenanthrene. This result is expected, as pH normally does not affect neutral analyte retention in reversed-phase HPLC [25], and serves as further confirmation that the anion-exchange column behaves in a typical reversed-phase manner.

The results for some smaller and polar analytes at low methanol concentration are another matter (Fig. 4B). This study was done over a wider pH range in a methanol–water (10:90), 2 mM KHP mobile phase. Chloroethylbenzene and ethylbenzene exhibit a *k* decrease across the entire pH range studied. The *k* for acetophenone decreases up to about pH 3.5, then levels out, while that of *p*-cresol decreases slightly up to pH 3.5, then increases, resulting in a retention order reversal with acetophenone. Because these results seemed unusual the experiments around pH 2, 4, 5 and 7 were run three times with three injections each, in random order, with mobile phase made up fresh each time. The absolute standard deviations of these four points are given on Fig. 4B, confirming a retention order reversal for *p*-cresol and acetophenone around pH 4, where the two analytes coelute.

To attempt to shed some light as to the nature of these pH effects two changes were made in the experiment. First, the four analytes were run in the same mobile phase (methanol–water (10:90), 2 mM KHP) but with a phenyl column replacing the anion-exchange phase. The idea here is that a propylphenyl group serves as the backbone for the anion-exchange phase so that this experiment simply leaves off the ammonium ionophore site. The results shown in Fig. 5A are typical reversed-phase behavior, no pH effect.

The second change was to employ an acetate mobile phase instead of the KHP on the anion-exchange column. These results, in methanol–water (10:90), 2 mm acetate, are shown in Fig. 5B. Ethylbenzene, chloroethylbenzene and acetophenone show little or no change in retention over the pH 3–7

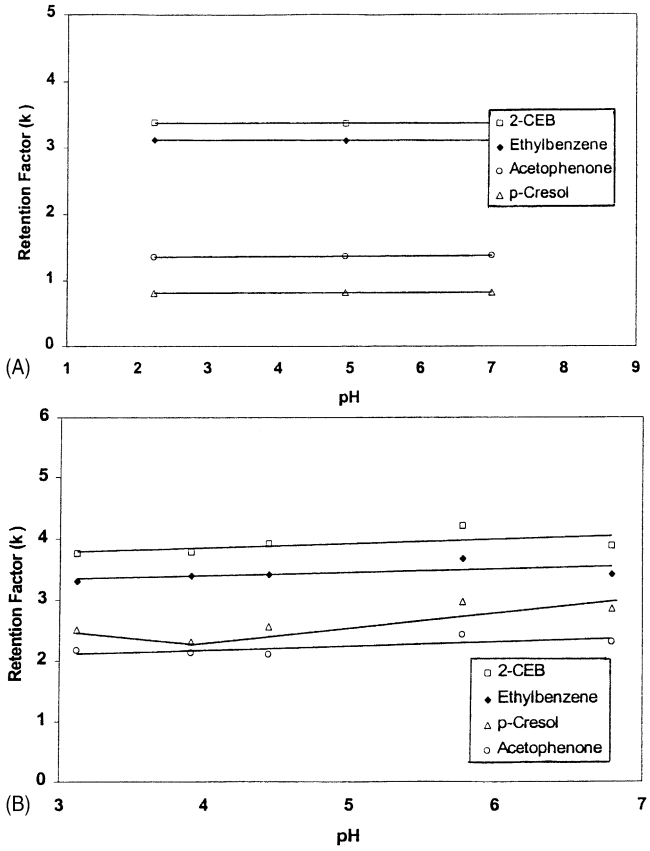

Fig. 5. Effect of pH on the retention factor of neutral organic compounds. (A) Phenyl column; mobile phase: methanol–water (10:90, v/v), 2 mM potassium hydrogen phthalate. (B) Strong anion-exchange column; mobile phase: methanol–water (10:90, v/v) 2 mM acetate. CEB is 2-chloroethylbenzene.

range. The behavior of *p*-cresol is very similar to that on the anion-exchange column with 2 mM KHP eluent, although no retention order reversal with acetophenone occurs. While these experiments were somewhat inconclusive, it appears that the combination of the anion-exchange phase and the phthalate eluting ion does result in changes in retention with pH for some organic analytes. Clearly more work needs to be done to sort out these effects.

3.1.5. Effect of pH on anion retention

The effect of pH changes on anion retention is well known [26]. In the broadest sense the charge of the stationary phase exchange site; of the analyte; and of the eluting ion can be affected by a change in pH. As this study used a strong anion-exchange phase the ionophore is positively charged across the entire pH range examined, as noted above. In addition, we chose anions of strong acids (Cl^-, NO_3^-) as analytes to preclude a change in analyte charge. This leaves the eluting ion, phthalate in our case, as having an effect on anion retention with a change in pH. The results are what would be expected as the pH is taken to the basic side of pK_{a2} for phthalic acid (5.5 in 100% aqueous solution). As the mobile phase gets richer in the doubly charged phthalate ion the eluent becomes stronger and *k* decreases, for example, from about 20 at pH 5.5 to about 4 at pH 7 for nitrate ion. So conventional anion-exchange behavior is found even in a mobile phase that is also appropriate for reversed-phase chromatography (methanol–water (60:40), 5 mM phthalate).

3.2. Some example separations

As noted earlier, separations of mixtures of anions and neutral organics were done both in relatively high methanol–content mobile phases, in which PAHs were the test analytes, and in low-methanol eluents in which polar organics were studied. This was done to examine the reversed-phase utility of the mixed-mode anion-exchange phase over a wide range of methanol concentration and neutral analyte polarities, and to assess the efficacy of combined ion-neutral determinations.

Separations of PAHs and anions were attempted in a mobile phase consisting of methanol–water (70:30), 5 mM KHP, pH 7.5. Overlap of the two analyte classes occurred allowing potential interference. Clearly, it was desirable to move the anions out to longer retention times and the PAHs to shorter retention times to eliminate class overlap. Knowing that the PAHs observe typical reversed-phase behavior the methanol content was increased to 75% (v/v). In addition, the pH of the mobile phase was decreased from 7.5 to about 6.6 to increase retention of the anions. The result is shown in Fig. 6, where the PAHs elute as a group in less than 10 min and the anions, also as a group, elute with retention times above 12 min.

A similar situation was found for the separation of polar organics and anions. Overlap of the neutral organics and anions occurred in a 15% (v/v) methanol mobile phase, but

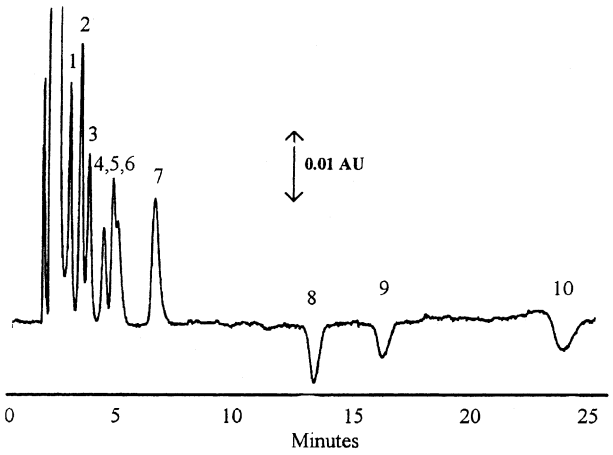

Fig. 6. Simultaneous separation of PAHs and inorganic anions on a strong anion-exchange column. Mobile phase: methanol–water (75:25, v/v), 5 mM potassium hydrogen phthalate, pH 6.6. Analytes: (1) phenanthrene, (2) pyrene, (3) chrysene, (4) benzo[*k*]fluoranthene, (5) benzo[*a*]pyrene, (6) perylene, (7) benzo[*ghi*]perylene, (8) iodate, (9) chloride, (10) nitrate. Detection: UV at 301 nm.

in 20% methanol the four organic analytes eluted as a group in less than 7 min and the five anions as a group above 8 min. Of course, the anions could be made to elute even later if the pH were also lowered. Clearly, small changes in methanol content or pH can be used to fine-tune these separations.

Many food, beverage and pharmaceutical samples contain organic and inorganic constituents that are amenable to simultaneous separation on an ion-exchange column [4]. An example of this is shown in Fig. 7, the chromatographic analysis of a Merlot red wine. The class separation described above between organics and anions is clearly seen here.

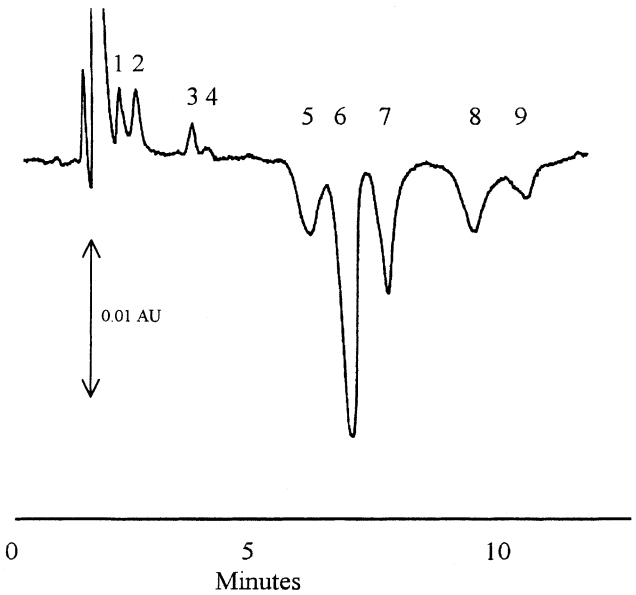

Fig. 7. Chromatogram of a Merlot red wine on a strong anion-exchange column. Mobile phase: methanol–water (10:90, v/v), 2 mM potassium hydrogen phthalate, pH 5.0. Analytes: (1–4, 7, 9) unknown, (5) lactate, (6) acetate, (7) chloride. Detection: UV at 260 nm.

While we did not attempt to identify all the peaks, the neutral organics were eluted in less than 5 min and the anions as a group above 5 min in a methanol–water (10:90) 2 mM KHP mobile phase.

3.3. Column efficiency and figures of merit

In order to further illustrate the efficacy of using a strong anion-exchange phase for simultaneous anion–neutral organic separations some data on column efficiency and the reproducibility of peak area and retention factor were obtained, along with some calibration curve results.

In general, efficiencies on the strong anion-exchange columns were better for anions than for neutral organic species. Plate counts for anions were generally in the 30–40,000 plates per meter range, while those for the organics were in the 12–20,000 plates per meter vicinity. While the efficiencies for the inorganic anions are typical, those for the organic analytes are low by normal reversed-phase standards. Of course this stationary phase was designed to be used for anions and so was not optimized for reversed-phase work.

Retention factors were obtained from three injections of a particular analyte, repeated over a 3-day period, during which a variety of mobile phases was used. The column was cleaned each day. The standard deviations of k were in the 1–3% (relative) range in the 60–70% (v/v) methanol–aqueous mobile phases and in the 1–7% (relative) range in the 10% (v/v) methanol–aqueous mobile phase. Peak areas for a series of three to four injections generally had standard deviations of 1% (relative) or less. Calibration curves using peak areas were obtained for pyrene, phenanthrene, chloride and nitrate. Slopes of the log of peak area versus log of the analyte concentration were in the expected 0.9–1.0 range for all four analytes. The linear dynamic range extended for three orders-of-magnitude.

4. Conclusions

While efficiencies of the mixed-mode separations shown here are acceptable for anions, as expected, they are only modest for neutral organic analytes. This is not surprising as the anion-exchange stationery phase was not optimized for reversed-phase work. A similar pattern was observed on a strong cation-exchange phase [4]. Still, the work described here demonstrates the proof-of-concept that mixtures of neutral organic and inorganic anion analytes can be simultaneously determined, and that the reversed-phase and ion-exchange interactions occur essentially independently of each other. The ability to separate neutral organics and anions both between and within classes using pH and organic cosolvent manipulation is particularly useful, and should allow rapid screening of complex samples followed by column switching downstream for more detailed separation or identification. The results of this study might also lead to the production of phases optimized for both ion-exchange and reversed-phase separation.

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