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Separation of carboxylates by hydrophilic interaction liquid chromatography on titania

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ABSTRACT

Hydrophilic interaction liquid chromatography (HILIC) can be performed on titania. To better understand the retention mechanisms on titania, a series of model carboxylates were used. Increasing acetonitrile above 60% dramatically increased the retention and efficiency for carboxylates. The effect of buffer type, buffer concentration, buffer pH and column temperature were also studied. Multiple retention mechanisms are operative on titania, and whether electrostatic repulsion, ligand exchange or HILIC dominates retention and separation depends on the eluent conditions. Guidelines for separations on titania are: (1) higher %ACN most improves retention and efficiency; (2) higher salt concentration increases retention; (3) elution strength is in the order acetate ≪ malate < methyl phosphonate ≪ phosphate; (4) electrostatic repulsion (ERLIC) is more operative at low %ACN than high %ACN. A bare titania column (150 mm × 4.6 mm I.D., 5 μm) was used for the separation of diphenylacetate, 4-nitrobenzoate, benzoate, 4-aminobenzoate, 4-hydroxybenzoate, phthalate, 3-aminophthalate, 1,3,5-benzenetricarboxylate, 1,2,4-benzenetricarboxylate, 1,2,4,5-benzenetetracarboxylate, benzenepentabenzoate and mellitate under HILIC conditions based on these guidelines, with efficiencies of 2800–55,000 plates/m.

1. Introduction

Titania, as a new packing material for HPLC, has drawn increasing attention in recent years. It has better mechanical and pH stability than silica [1,2]. Titania is an anion exchanger at low pH and a cation exchanger at a high pH [3,4]. Due to the unsaturated Lewis acids sites on the titania surface, it also behaves as a ligand exchanger with Lewis bases [5,6] such as phosphates. Thus titania has been used by several groups to concentrate nucleotides and phosphopeptides [7–11]. In addition, titania-based HPLC columns have good temperature stability up to 160 °C [12] which can be used in high temperature liquid chromatography.

Previously, we separated 13 nucleotides and their pathway intermediates on a bare titania column with resolution ≥1.3 [13]. With high concentrations of acetonitrile in the eluent, hydrophilic interaction liquid chromatography is the dominant mode of retention. The term hydrophilic interaction liquid chromatography (HILIC) was coined by Alpert to emphasize a unique retention behavior on polar liquid chromatographic columns [14]. HILIC implies the use of a polar stationary phase and a less polar mobile phase, normally containing a high percentage of an organic modifier such as acetonitrile (ACN) added to an aqueous solution. The elution order in HILIC is from least to most polar analyte—the reverse of that observed in RPLC. HILIC is considered a variant of normal phase chromatography that uses a "reversed phase" eluent, in which water is the stronger eluent and the retention mechanism is partitioning. When a high percentage of organic modifier such as ACN is added to the eluent, a water-enriched layer is formed on the hydrophilic stationary phase. Analytes partition between the water-enriched layer and the bulk hydrophobic eluent.

Compared to NPLC or RPLC, HILIC has several advantages for the separation of polar compounds. In RPLC polar analytes are weakly retained and thus cannot easily be resolved. In contrast, polar compounds are well retained on HILIC. In NPLC, water must be rigorously eliminated from the eluent. The high surface activity and acidity of silica makes it susceptible to traces of moisture. However in HILIC mode, water acts as a pseudo-stationary phase. Thus there is no need to eliminate water from HILIC eluents.

In this paper we investigate the retention of a series of carboxylates via HILIC on titania. The effect of %ACN, buffer type and concentration, pH and temperature are studied to better understand the retention mechanisms on titania. Basic guidelines governing HILIC retention on titania are established and used to develop separations for a number of carboxylate mixtures.

2. Experimental

2.1. Reagents

All solutions were prepared in nano-pure water (Barnstead, Dubuque, IA, USA). Sodium fluoride and HPLC-grade acetoni-

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trile (ACN) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sodium phosphate monobasic and sodium phosphate dibasic were from EM Science (Gibbstown, NJ, USA). Acetic acid was purchased from Anachemia (Rouses Point, NY, USA). Sodium acetate was from EMD (Darmstadt, Germany). Adenosine-5′-monophosphate, malic acid, methylphosphonic acid, benzoic acid, diphenylacetic acid, 4-aminobenzoic acid, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, phthalic acid, 3-aminophthalic acid, 1,2,4-benzenetricarboxylic acid, 1,3,5-benzenetricarboxylic acid, 1,2,4,5-benzenetetracarboxylic acid, benzenepentacarboxylic acid and mellitic acid (benzenehexacarboxylic acid) were from Sigma–Aldrich (St. Louis, MO, USA). p-Nitrobenzoic acid was from Eastman (Rochester, NY, USA). All solutions were filtered with 0.22 μm Magna nylon membrane filters (GE Osmonic, Trevose, PA, USA) prior to use.

Eluents were prepared from 1000 mM stock solutions (from pH 6 to 10). To prepare the 1000 mM stock solution, a small amount of sodium hydroxide was added to adjust the buffer pH. The necessary volume of stock solution was first added to the volumetric flask, followed by the adding of ACN and then water was added till the calibration mark. The buffer concentrations quoted in this paper are those present after ACN addition and are the total concentration of phosphate in solution. The dead time was measured based on the solvent front peak. Buffer pH was adjusted by a Corning combination 3-in-1 electrode (Corning, Big Flats, NY, USA) before adding ACN. All samples were dissolved in the same %ACN as the eluent [13].

2.2. Chromatographic conditions

Separations were performed using a model 709 dual-piston pump (Metrohm, Herisau, Switzerland) operating at 1.0 mL/min, a 6-port Cheminert CCP0140 injection valve with a 20 μL loop (Valco Instruments, Houston, TX, USA), and a Lambda-Max Model 481 UV detector at 254 nm (Waters, Milford, MA, USA). Data was collected at 30 Hz using a Metrohm 762 data acquisition system with IC Net 2.1 software. A bare TiO_2 Sachtopore-NP column (150 mm × 4.6 mm I.D., 5 μm, 300 Å, 15 m^2/g, Zirchrom, Anoka, MN, USA) was used.

In studies of the effect of temperature, the titania column was placed in an Eppendorf CH-30 column heater controlled by an Eppendorf TC-50 (Westbury, NY, USA). Upon changing the temperature, the column was allowed to equilibrate for 15 min before making any measurements. The column equilibration time is defined as the time necessary to achieve stable retention after a 10 °C increase in the column temperature.

3. Results and discussion

3.1. %ACN

HILIC separations of carboxylates have been reported on a variety of columns including NH_2, amide, zwitterionic (ZIC) and silica [15–19]. On these columns the retention of carboxylates increased with increasing %ACN when >60% ACN was present in the eluent [15,16,18,19]. To test whether carboxylates will follow the same retention trend on titania, 5 mM pH 6.0 sodium phosphate buffer in different %ACN was used as the eluent. (All buffer concentrations quoted are the final concentration of phosphate after addition of ACN.) Analytes were dissolved in the same %ACN as the eluent to avoid injection solvent induced broadening [13,20,21]. Fig. 1 shows the effect of %ACN on the retention and efficiency of 1,2,4-benzenetricarboxylate and 1,2,4,5-benzenetetracarboxylate. Retention barely increases with increasing %ACN from 0 to 60%, followed by a dramatic increase when %ACN is above 60%. This is consistent with what have been reported on other HILIC columns [15–19]. Also, when the eluent contained less than 60% ACN, the retention factors for 1,2,4-benzenetricarboxylate and 1,2,4,5-benzenetetracarboxylate were below 0 (inset in Fig. 1a) due to electrostatic repulsion between titania and the carboxylates (discussed in detail in Section 3.2).

Fig. 1b shows that for 0–50% ACN efficiency is essentially low and constant. Above 60% ACN there is a great increase in efficiency: from 14,000 to 78,000 plates/m for 1,2,4-benzenetricarboxylate for 60–80% ACN; and 23,000–80,000 plates/m for 1,2,4,5-benzenetetracarboxylate for 60–75% ACN. In comparison efficiencies for carboxylates on the YMC-Pack NH_2, TSKgel Amide-80, ZIC-HILIC and silica HILIC columns range from 5000 to 70,000 plates/m at 85% ACN [15]. Thus the titania column provides comparable to better efficiency to commercial HILIC columns.

3.2. Buffer type and concentration

Previously phosphate was used as the eluent buffer for HILIC separations of nucleotides on a bare titania column [13]. The phosphate buffer saturated the ligand exchange sites on titania enabling HILIC retention to dominate when the eluent contained high %ACN. Buffers such as acetate and methylphosphonate were too weak to elute the nucleotides. Carr and co-worker [22] reported that carboxylates have a weaker ligand affinity with zirconia than organophosphates and much weaker than phosphate. The retention behavior of titania is similar to that of zirconia [5,22]. Herein it should be possible to use a weaker eluent such as acetate to saturate the active sites on titania and enable carboxylates to be separated in the HILIC mode.

To find the appropriate buffer type and concentration, varying concentrations of sodium phosphate, sodium acetate, sodium methylphosphonate and sodium malate at pH 6.0 in 75% ACN were tested as eluents (Fig. 2). For a given buffer concentration, sodium phosphate is the strongest eluent on titania, followed by sodium methylphosphonate and sodium malate, and sodium acetate is the weakest eluent. This is consistent with the eluotropic series for zirconia [22].

For all eluent buffers, retention increases with increasing buffer concentration (Fig. 2a). Increased retention with the increasing salt concentration has been reported frequently on a variety of HILIC columns [15,16,18,23–28], but no definitive mechanism for this phenomenon has been established. Two possible causes have been hypothesized. Firstly, higher salt concentration might drive the more solvated salt ions into the water-enriched layer formed on the particles, yielding an increase in the volume of the water layer and therefore an increase in retention [15,24,25,28]. Secondly, electrostatic repulsion between the stationary phase and the analytes is weakened by the higher salt concentrations [18,24–26]. These two theories will be discussed in detail in Sections 3.2.1 and 3.2.2.

Fig. 2b shows the effect of buffer concentration on the chromatographic efficiency. The efficiency increases with increasing buffer concentration for all buffers except sodium acetate. The increase in efficiency with increasing buffer concentration in Fig. 2b is much less dramatic than that when increasing %ACN (Fig. 1b). For example, the efficiency for 1,2,4-benzenetricarboxylate increased from 15,000 to 80,000 plates/m when increasing the %ACN from 60 to 80% with 5 mM sodium phosphate buffer in the mobile phase. However, it only increased from 50,000 to 70,000 plates/m when increasing sodium phosphate concentration from 5 to 10 mM in 75% ACN.

3.2.1. Water layer on titania

McCalley and Neue concluded that about 4–13% of the pore volume of a silica phase is occupied by a water-enriched layer when 75–90% ACN is present in the eluent [29]. Hydrophobic analytes

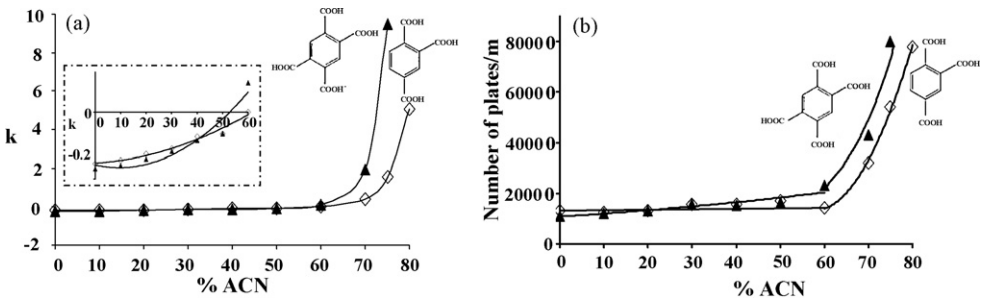

Fig. 1. Effect of acetonitrile concentration on (a) retention factor (k) and (b) efficiency (N) on titania. Conditions: flow rate, 1.0 mL/min; eluent, 5 mM sodium phosphate buffer (pH 6.0) in 0–80% (v/v) ACN; analyte, 0.1 mM 1,2,4-benzenetricarboxylic acid (◊) and 1,2,4,5-benzenetetracarboxylic acid (▲) in the same %ACN as the eluent; UV detection at 254 nm. Line is a guide to the eye.

such as toluene do not undergo ligand exchange or electrostatic interactions, and due to their hydrophobic nature are excluded from the water layer. Thus the retention time of toluene with ACN/water eluents reflects the void volume of the column minus the volume of the water layer. Bicker et al. indirectly monitored the volume of the water layer under HILIC conditions by measuring the difference in the retention time of toluene when the protic modifier was changed from water to methanol [28]. The retention time of toluene should change due to the water layer being replaced by a methanol layer of a different thickness and to the increased partitioning of toluene into the more hydrophobic alcohol layer.

To confirm the presence of a water-enriched layer on titania under the conditions used herein, a similar experiment was performed. When H_2O in 80% ACN was replaced with methanol the retention time of toluene increased from 1.62 ± 0.00 to 1.67 ± 0.00 min (3% increase). Bicker et al. observed a 10% increase in retention time on silica to which oxidized 1-thioglycerol was bonded [28]. Considering the huge difference in surface area between their silica (300 m^2/g) and the titania used herein (15 m^2/g [6]), the increase in the void time confirms the presence of a water on titania and suggests that it is thicker than on silica.

The effect of buffer concentration on the water layer was also monitored in this manner. The retention time of toluene decreased from 1.62 ± 0.00 to 1.57 ± 0.00 min (3% decrease) when the concentration of sodium acetate (pH 6.0) was increased from 0 to 20 mM in 80% ACN. This decrease indicates that the volume of water layer on titania increases when increasing buffer concentration, consistent with the increase in polar analyte retention observed in Fig. 2a.

3.2.2. Electrostatic repulsion on titania

For HILIC columns with charged surfaces, the surface charge affects retention of charged analytes [18,24,26,30]. Retention of oppositely charged analytes increases due to ion exchange, while analytes with the same charge as the stationary phase experience decreased retention due to electrostatic repulsion. At low % organic solvent, analytes with the same charge as the stationary phase elute prior to the void time as Donnan exclusion prevents the analytes from accessing the stationary phase within the pores [30]. However, when the mobile phase contains higher % organic solvent, hydrophilic interaction ensures that the charged analytes are retained well despite the electrostatic repulsion. Alpert defined the term electrostatic repulsion hydrophilic interaction chromatography (ERLIC) to describe the combination of electrostatic repulsion and HILIC [30] which occurs at high % organic modifier with columns of the same charge as the analytes.

Titania has an isoelectric point of 5.0–5.6 [31,32]. It acts as an anion exchanger (positively charged surface) at low pH or a cation exchanger (negatively charged surface) at high pH [3,4]. The studies above were performed at pH 6.0 (measured before adding ACN to the eluent). However, the high %ACN used here makes it difficult to state whether the conditions are above or below the isoelectric point of titania. The negative retention factor of carboxylates at 0–50% ACN (Fig. 1a) indicates that the titania was negatively charged. However, above 60% ACN, the k of the carboxylates becomes positive (Fig. 1a), which shows that electrostatic repulsion is overwhelmed by another retention mode such as hydrophilic interaction. Thus with the high %ACN (75%) in Fig. 2, electrostatic repulsion is no longer dominant.

There is another phenomenon which draws our interest in Fig. 2. The eluent strength increased in the order of acetate < malate < methylphosphonate < phosphate. This is consistent with the eluotropic series that Carr and co-workers observed on zirconia [22]. In their eluotropic series, phosphate is the strongest ligand exchanger for eluting benzoate derivatives, organophosphate (methylphosphonate in our case and ethylphosphonate in their series) and malate are a little bit below phosphate, and acetate acts as a much weaker eluent. The same eluent strength order in Fig. 2a as the eluotropic series indicates that the active ligand exchange sites on titania are not yet fully saturated by the eluent anion.

As mentioned before, electrostatic repulsion is dominant at low %ACN, which keeps the charged analytes away from the titania

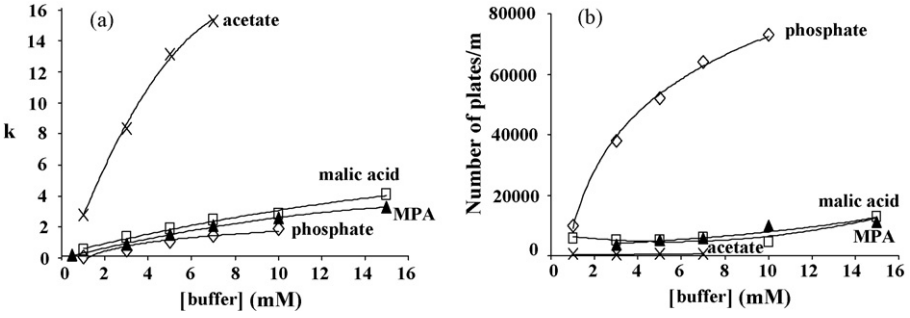

Fig. 2. Effect of buffer type and concentration on retention factor (k) on titania. Conditions: flow rate, 1.0 mL/min; eluent, salt solution (pH 6.0) in 75% (v/v) ACN; analyte, 0.1 mM 1,2,4-benzenetricarboxylic acid in 75%ACN; UV detection at 254 nm.

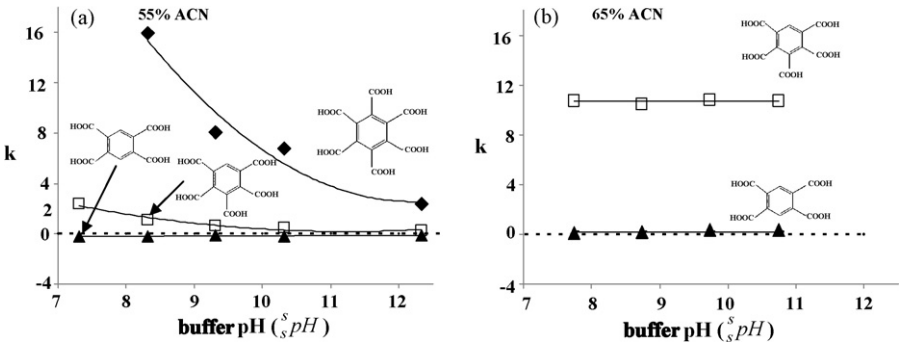

Fig. 3. Effect of buffer pH on retention at different %ACN. Conditions: flow rate, 1.0 mL/min; eluent, 3 mM sodium phosphate buffer in (a) 65% ACN and (b) 55% ACN; analyte, 0.1 mM 1,2,4,5-benzenetetracarboxylic acid (▲), 1,2,3,4,5-benzenepentacarboxylic acid (□), mellitic acid (×) dissolved in the same %ACN as the eluent; UV detection at 254 nm. Lines are guides for the eye. Dashed lines are drawn at $k = 0$.

surface. With high %ACN present, a water layer is formed on the stationary phase, which diminishes the electrostatic repulsion and draws the hydrophilic analytes closer to the surface. This closer space between the analytes and the titania surface makes it possible for ligand exchange to occur.

At low buffer concentrations, the ligand exchange sites on titania surface are not saturated. Thus the analytes can undergo ligand exchange with the titania surface. This explains the poor efficiency when using sodium acetate as the buffer solution (Fig. 2b), as ligand exchange on titania displays poor efficiency [33–35]. However, if ligand exchange is the dominant retention mode on titania under this eluent condition, retention should decrease when increasing the buffer concentration, which is contrary to the behavior observed in Fig. 2a. Once the surface is significantly modified by a strong ligand exchange buffer such as phosphate, hydrophilic interaction becomes the dominant retention mode, resulting in increasing retention and efficiency with the increasing buffer concentration.

From the above, it is concluded that the retention behavior of carboxylates on titania surface has a mixed-mode mechanism. HILIC, electrostatic repulsion and ligand exchange all contribute to retention, with the mobile phase conditions determining which mode is dominant. At low %ACN, electrostatic repulsion is dominant, resulting in the negative k of carboxylates on titania. At high %ACN (above 60%), electrostatic repulsion is diminished by the formation of the water layer on titania. With high %ACN in the mobile phase, low concentrations of a weakly associating buffer do not fully saturate the ligand exchange sites on titania. Thus ligand exchange still takes effect, and poor efficiencies are observed. High concentrations of medium-strong associating buffers with high %ACN modifies the ligand exchange sites on titania, allowing hydrophilic interaction to be dominant and provide high efficiency.

3.3. Buffer pH

3.3.1. Background

When an organic modifier is added to the analyte and eluent buffer solution, there is a change in the pK_a of both the analyte and the eluent. Consequently, the pH of the eluent and the degree of ionization of the analyte also change [36–39]. These variations may result in significant changes in retention and selectivity in HPLC. Roses and co-workers have extensively studied the estimation and calculation of pH and ionization upon adding organic modifiers such as methanol and acetonitrile to aqueous solutions. They defined several terms to illustrate the pH values under various calibration and measurement conditions [37]. $^{w}_{w}$pH is the pH value when the electrode is calibrated with aqueous buffers and pH is measured in aqueous buffers before mixing with the organic modifier. $^{s}_{s}$pH is measured in the eluent buffers containing organic modifier with electrodes calibrated in buffers with the same % organic modifier as the mobile phase. The pH values shown in the experimental conditions in this paper are all $^{w}_{w}$pH except for Fig. 3 which uses $^{s}_{s}$pH.

Eq. (1) shows the relationship between $^{w}_{w}$pH and $^{s}_{s}$pH, where δ is a constant for each mobile phase composition, m_{pH} is the proportionality coefficient for the pH change and ϕ_{ACN} is the volume fraction of the organic modifier (ACN) [39]:

$$^{s}_{s}\mathrm{pH} = {}^{w}_{w}\mathrm{pH} + m_{\mathrm{pH}} \times \phi_{\mathrm{ACN}} - \delta \quad (1)$$

As mentioned before, the pK_a of analytes is also changed when dissolved in organic modifier. Eq. (2) shows the pK_a change when changing the fraction of organic modifier in the dissolving solvent, where a_s and b_s are the fitting constants for acids of the same family [39]:

$$^{s}_{s}\mathrm{pK_a} = a_s {}^{w}_{w}\mathrm{pK_a} + b_s \quad (2)$$

3.3.2. Effect of pH on retention

Fig. 3 shows the effect of buffer pH on retention in 55% and 65% ACN. The pH shown in Fig. 3 are $^{s}_{s}$pH calculated using Eq. (1) with m_{pH} and δ from Ref. [39] (m_{pH} is from 1.777 to 1.810 depending on $^{w}_{w}pH$ and remains the same at different %ACN; $\delta = -0.33$ at 55% ACN and -0.58 at 65% ACN). In 55% ACN (Fig. 3a), the retention factor of 1,2,4,5-benzenetetracarboxylate is always negative and does not change significantly when changing buffer pH. Retention of benzenepentacarboxylate and mellitate decreases asymptotically to near zero with increasing pH. At 65% ACN in Fig. 3b, changing buffer pH from 7.7 to 10.7 does not change the retention of the carboxylates. For the same buffer pH and concentration, lower %ACN results in a lower retention, which is consistent with Fig. 1a. For example, mellitate cannot be eluted in 30 min in 65% ACN. With 55% ACN it is eluted from titania and the retention factor drops 85% when increasing buffer pH from 7.3 to 12.3. The retention for the early eluted 1,2,4,5-benzenetetracarboxylate did not change a lot when changing buffer pH in both 55% and 65% ACN. However, it is eluted prior to the void time in 55% ACN and starts to have a positive retention factor in 65% ACN.

There is an increase in efficiency upon increasing buffer pH in both 65% and 55% ACN. For example increasing the buffer pH from 7.7 to 10.7 increased the efficiency for benzenepentacarboxylate from 2000 to 9000 plates/m in 65% ACN and from 100 to 3000 plates/m in 55% ACN. The higher efficiency in higher %ACN is consistent with the general behavior observed in Fig. 1b.

Based on the above observations, for the separation of late-eluted analytes such as benzenepentacarboxylate and mellitate, a high buffer pH in lower %ACN yields faster separation, albeit with lower efficiency. At higher %ACN buffer pH has no effect on retention.

Table 1
pKa change of carboxylic acids when changing %ACN in solvent.

Analyte	pK_a in 0% ACN [40]	${}^{s}_{s}pK_a$ in 55% ACN[a]	${}^{s}_{s}pK_a$ in 65% ACN[a]
1,2,4,5-Benzenetetracarboxylic acid	1.92, 2.87, 4.49, 5.63	2.74, 4.11, 6.45, 8.09	3.07, 4.56, 7.10, 8.89
1,2,3,4,5-Benzenepentacarboxylic acid	1.80, 2.73, 3.97, 5.25, 6.46	2.57, 3.91, 5.70, 7.54, 9.29	2.88, 4.34, 6.29, 8.30, 10.19
1,2,3,4,5,6-Benzenehexacarboxylic acid (mellitic acid)	1.40, 2.19, 3.31, 4.78, 5.89, 6.96	1.99, 3.13, 4.74, 6.86, 8.46, 10.01	2.78, 3.49, 5.25, 7.56, 9.30, 10.98

[a] Calculated from Eqs. (11) and (12) from Ref. [39].

At 55% ACN (Fig. 3a), there is an increase in ionization for all three carboxylates with increasing pH. At pH 7.31, all three carboxylates are not fully ionized according to their pK_a values (Table 1). However the ionization degree is different. The ionization of 1,2,4,5-benzenetricarboxylate (α_4 = 13%) is much higher than that of benzenepentacarboxylate (α_5 = 0.15%) and mellitate (α_6 = 0.01%). Increasing buffer pH thus will not dramatically change the ionization of 1,2,4,5-benzenetetracarboxylate and the electrostatic repulsion, resulting in constant retention. The ionization degree of benzenepentacarboxylate increases with the increasing buffer pH until pH 11 where it is 98% ionized. With buffer pH higher than 11, the ionization degree and the electrostatic repulsion are not changed, which explains the appearance of the plateau. Similar to benzenepentacarboxylate, mellitate has pK_{a6} of 10.01, which results in stable retention above pH 12 (99% ionized). Thus, the decreasing retention in Fig. 3a is consistent with increased electrostatic repulsion (ERLIC).

In Fig. 3b, retention of 1,2,4,5-benzenetetracarboxylate and benzenepentacarboxylate are unchanged by increasing the pH from 7.74 to 10.74 in 65% ACN. Table 1 shows the pK_a values (${}^{w}_{w}pK_a$ data from Ref. [40]) converted into ${}^{s}_{s}pK_a$ using Eqs. (11) and (12) in Ref. [39]) (a_s = 1.443, b_s = -0.032 in 55% ACN; a_s = 1.569, b_s = 0.058 in 65% ACN). Both 1,2,4,5-benzenetetracarboxylate and benzenepentacarboxylate are increasingly ionized over this pH range. Fig. 3b does not agree with either pure HILIC (greater retention [15]) or ERLIC (lower retention [30]). Thus at 65% ACN both HILIC and ERLIC are operative and counter-balance one another.

Herein it is concluded that at lower %ACN (55%) electrostatic repulsion has a significant effect on the retention. Increasing ionization decreases the retention as the electrostatic repulsion is increased. The lower electrostatic repulsion observed in Fig. 3b is consistent with the lower repulsion observed at higher %ACN (Section 3.2.2). This also explains the negative retention factor of 1,2,4,5-benzenetetracarboxylate at 55% ACN and a positive retention factor at 65% ACN.

3.4. Column temperature

High column temperature (HTLC) offers reduced back pressure, faster separations [41–47] and higher efficiency [48,49]. Titania is stable up to 160 °C while ordinary silica is stable only to 60–70 °C even at neutral pH [6]. Thus zirconia or titania-based columns have been widely used for high temperature separations in both the reversed and normal phase modes [12,48,50–52]. Given the thermal stability of titania, it was of interest to explore the temperature behavior of HILIC on titania.

Contrary to the behavior observed in RPLC, retention increased with temperature on titania using a 5 mM phosphate buffer (pH 6.0) in 75% (v/v) ACN. For instance, k for 1,2,4,5-benzenetetracarboxylate increased from 3.7 to 4.3 and AMP from 0.6 to 0.7 upon increasing the column temperature from 30 to 70 °C. The effect of column temperature in HILIC has been discussed extensively in the literature, with both increasing [53–57] and decreasing [15,18,23,25–28,58,59] retention being observed. Van't Hoff plots ($\ln k$ vs. $1/T$ (column temperature)) were linear (R^2 = 0.97) for both 1,2,4,5-benzenetetracarboxylate and AMP. Again the literature is ambiguous as both linear and nonlinear [18,58] Van't Hoff plots have been observed for HILIC. Regardless, enthalpies (ΔH) determined from the Van't Hoff plots are statistically equivalent for AMP and 1,2,4,5-benzenetetracarboxylate, 3.1 ± 0.2 and 3.4 ± 0.2 kJ/mol respectively. The equivalence of these enthalpies is not consistent with direct analyte interaction with the titania surface, as organophosphates undergo much stronger ligand exchange than carboxylates [22]. The greater retention of 1,2,4,5-benzenetetracarboxylate than AMP in Fig. 4 also suggests that ligand exchange is not occurring. The magnitude of these enthalpies also indicates that the analytes are not in direct contact with the titania surface. The desorption ΔH between titania and physisorbed water is 51 kJ/mol [60], which is much higher than the ΔH values observed herein. Thus the Van't Hoff results indicate the analytes are most likely partitioned into the water layer on titania [53], i.e., are retained by a HILIC mechanism.

From 30 to 70 °C the efficiency of 1,2,4,5-benzenetetracarboxylate increased from 23,000 to 30,000 plates/m and that of AMP increased from 5000 to 6000 plates/m. However increased temperature resulted in an increased column equilibration time; 48 ± 3 min was needed to equilibrate at 30 °C whereas 78 ± 3 min was necessary at 60 °C. Given the small improvement in efficiency and longer column equilibration time necessary with higher column temperature, no net advantage to performing experiments at elevated temperature were achieved. Therefore room temperature was used for all the future studies.

3.5. Separation of carboxylates

Based on the above discussion, guidelines for separations of carboxylates on bare titania can be drawn: (1) higher %ACN is the most helpful for improving retention and efficiency; (2) higher buffer concentration increases retention; (3) the elution strength of buffers is acetate ≪ malate < methyl phosphonate < phosphate; (4) electrostatic repulsion (ERLIC) is more operative at low %ACN than high %ACN. These guidelines were used to optimize the eluent conditions in the following separations.

3.5.1. Mono-carboxylates

Fig. 5 shows the separation of a series of mono-carboxylates. An eluent of 10 mM acetate (pH 6.0) in 95% ACN was used. Increasing the %ACN from 85 to 95% with 10 mM acetate pH 6.0 buffer in the mobile phase resulted in a 40% increase in the critical pair resolu-

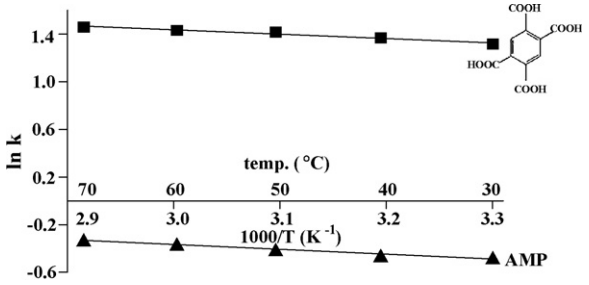

Fig. 4. Effect of temperature on retention on titania. Conditions: flow rate, 1.0 mL/min; eluent, 5 mM $H_2PO_4^-$/HPO_4^{2-} buffer (pH 6.0) in 75% (v/v) ACN; analyte, 0.1 mM AMP and 1,2,4,5-benzenetetracarboxylic acid in 75%ACN; UV detection at 254 nm. Lines are fits to Eq. (5).

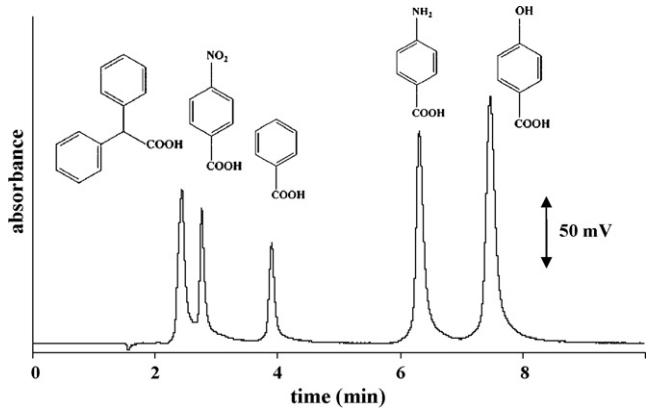

Fig. 5. Separation of mono-carboxylates on titania. Conditions: flow rate, 1.0 mL/min; eluent, 10 mM sodium acetate buffer (pH 6.0) in 95% (v/v) ACN; analyte, 0.06–1 mM of each carboxylate in 95%ACN; UV detection at 254 nm.

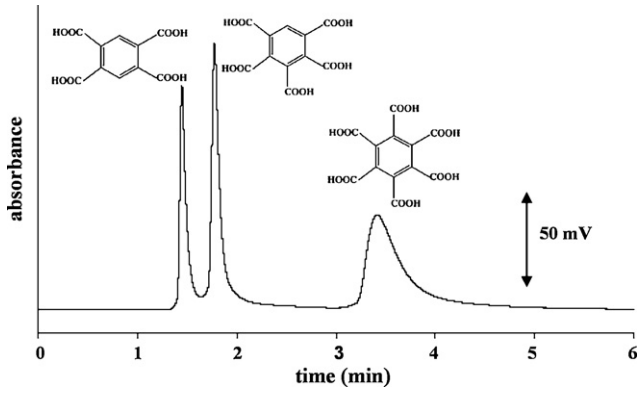

Fig. 7. Separation of multi-carboxylic acids on titania. Conditions: flow rate, 1.0 mL/min; eluent, 3 mM sodium acetate buffer (pH 11.0) in 50% (v/v) ACN; analyte, 0.1–0.25 mM of each carboxylate in 50%ACN; UV detection at 254 nm.

tion (from 1.2 to 1.6). Thus very high %ACN increased the retention and resolution as per rule 1 above. Alternately, increasing buffer concentration can also increase retention and resolution (rule 2). However high buffer concentration at low %ACN is not as effective as simply using high %ACN. For example for the analytes in Fig. 5, use of 60 mM sodium acetate pH 6.0 buffer in 75% ACN resulted in zero resolution between 4-aminobenzoate and 4-hydroxybenzoate, and the maximum resolution within the chromatogram was only 0.8.

3.5.2. Multi-carboxylates

For mono-carboxylates, acetate is a sufficiently strong eluent. However, acetate is too weak to elute multi-carboxylates. Thus a stronger eluent (rule 3), sodium phosphate, must be used to elute and separate the di- and tri-carboxylates, as shown in Fig. 6. One millimolar sodium phosphate buffer (pH 6.0) in 85% ACN was the optimal eluent. Increasing the %ACN was the most effective way to increase efficiency and resolution. Increasing %ACN from 80 to 85% ACN (1 mM phosphate pH 6.0 buffer) increased the resolution between the critical pair from 0 to 7. Higher buffer concentrations (rule 2) were not used due to the low solubility of phosphate in high %ACN. It is not known why the 1,3,5-benzenetricarboxylate peak is fronted. Lowering the analyte concentration (0.2–0.1 mM) had no effect on peak shape and efficiency and retention time shifted less than 2%, indicating that overloading is not a factor.

For the multi-carboxylates, increasing the buffer pH at low %ACN (rule 4) was necessary to decrease retention to a useful level. At pH 6 (either acetate or phosphate) strong retention was observed regardless of the %ACN or buffer concentration. Increasing the phosphate eluent (in 50% ACN) pH from 6 to 11 reduced retention. Fig. 7 shows the baseline separation of 3 multi-carboxylates in 5 min using 3 mM sodium phosphate (pH 11.0) in 50% ACN as the eluent. Similar to 1,3,5-benzenetricarboxylate, the peak of mellitate in Fig. 7 is also broadened. Again lowering the analyte concentration had no effect on peak shape.

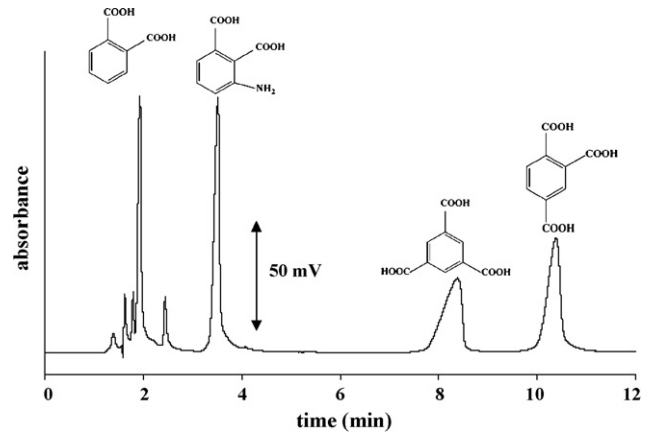

Fig. 6. Separation of di- and tri-carboxylates groups on titania. Conditions: flow rate, 1.0 mL/min; eluent, 1 mM sodium phosphate buffer (pH 6.0) in 85% (v/v) ACN; analyte, 0.1–0.2 mM of each carboxylate in 85%ACN; UV detection at 254 nm.

3.5.3. Elution order comparison

The elution order of carboxylates is compared here. For mono-carboxylates (Fig. 5), the eluent used was 10 mM sodium acetate (pH 6.0) in 95% ACN. As mentioned in Section 3.2, HILIC is the dominant retention mode at high %ACN. Diphenylacetate, with a higher hydrophobicity, elutes earlier than benzoate. 3,4-Dihydroxybenzoate elutes later than 4-hydroxybenzoate as its additional hydroxyl group makes it more hydrophilic. Similar elution has been observed on NH_2, amide, zwitterionic (ZIC), polyhydroxyethyl and silica HILIC columns [18]. Amine and hydroxyl groups can hydrogen bond with the water layer on titania surface, yielding stronger retention than p-nitrobenzoate.

For the tri-carboxylates (Fig. 6), 1,2,4-benzenetricarboxylate elutes after 1,3,5-benzenetricarboxylate even though they have very similar chemical structure. Alpert proposed that the orientation of an analyte may affect the electrostatic repulsion in ERLIC [14,30]. However, in this case the elution order more likely results from the ionization of the two isomers. The $pK_{a,3}$ are estimated (ACD/pK_a, v. 8.03, Advanced Chemistry Development, Toronto, ON, Canada) to be 5.20 ± 0.10 for 1,2,4-benzenetricarboxylic acid and 4.88 ± 0.10 for 1,3,5-benzenetricarboxylic acid. Thus 1,3,5-benzenetricarboxylic acid should be more ionized at pH 6.0 and so would be more repelled from the titania surface more than 1,2,4-benzenetricarboxylic acid, thus elutes earlier. So while HILIC is the dominant retention mode at the high %ACN (85%) used, electrostatic repulsion still has a significant effect. Similarly for the elution of p-nitrobenzoic acid and benzoic acid, p-nitrobenzoic acid elutes earlier (Fig. 5) due to the lower pK_a (3.41 for p-nitrobenzoic acid vs. 4.19 for benzoic acid [40]) and thus higher degree of ionization for p-nitrobenzoic acid. Hence, the separations are best thought of as ERLIC rather than a traditional HILIC mode.

The elution of multi-carboxylates follows a similar retention mechanism as the tri-carboxylates. A low %ACN (50%) is used to decrease the separation time. The elution order is 1,2,4,5-benzenetetracarboxylate ($^{s}_{s}pK_{a4} = 7.75$ [39]), benzenepentacarboxylate ($^{s}_{s}pK_{a5} = 8.91$ [39]), and mellitate ($^{s}_{s}pK_{a6} = 9.60$ [39]) at last. The negative retention factor of 1,2,4,5-benzenetetracarboxylate is due to the electrostatic repulsion.

Mellitate has the highest pK_a value, thus elutes last since it is the least ionized and has the least electrostatic repulsion with titania surface.

4. Conclusions

The retention behavior of carboxylates showed a mixed retention mechanism on titania. Both electrostatic repulsion and HILIC are involved. With high %ACN in the mobile phase, HILIC mechanism is dominant. Increasing buffer concentration and column temperature at high %ACN increased the retention factor of carboxylates. Increasing buffer pH decreased the retention factor when lower %ACN is added to the mobile phase. The separation of a series of carboxylates was achieved on a bare titania column by tuning the eluent strength.

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