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Cyclofructan 6 based stationary phases for hydrophilic interaction liquid chromatography

Haixiao Qiu[a], Lucie Loukotková[b], Ping Sun[a], Eva Tesařová[c], Zuzana Bosáková[b], Daniel W. Armstrong[a,*]

[a] Department of Chemistry and Biochemistry, The University of Texas at Arlington, Arlington, TX 76019, USA
[b] Department of Analytical Chemistry, Faculty of Science, Charles University in Prague, Prague, Czech Republic
[c] Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University in Prague, Prague, Czech Republic

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ABSTRACT

New stationary phases for hydrophilic interaction liquid chromatography (HILIC) were synthesized by covalently attaching native cyclofructan 6 (CF6) to silica gel. The chromatographic characteristics of the new stationary phases were evaluated and compared to three different types of commercial HILIC columns. The CF6 columns produced considerably different retention and selectivity patterns for various classes of polar analytes, including nucleic acid compounds, xanthines, β-blockers, salicylic acid and its derivatives, and maltooligosaccharides. Univariate optimization approaches were examined including organic modifier (acetonitrile) contents and buffer pH and salt concentration. The thermodynamic characteristic of the CF6 stationary phase was investigated by considering the column temperature effect on retention and utilizing van't Hoff plots. CF6 based stationary phases appear to have exceptionally broad applicability for HILIC mode separations.

1. Introduction

Among various separation modes in liquid chromatography, reversed phase liquid chromatography (RPLC) is by far the most frequently used. However, RPLC has limitations in the analysis of very polar compounds like sugars, amino acids, nucleosides, sulfonated compounds, etc. [1]. Such solutes typically have insufficient interactions with the hydrophobic surface of reversed phase materials and thus elute with poor retention and are often not separated from other polar compounds [2]. Over the last several years, hydrophilic interaction liquid chromatography (HILIC) has emerged as a viable alternative to RPLC for many applications. It has been steadily gaining popularity for the separation of polar and hydrophilic analytes, including carbohydrates [3,4], peptides [5], and polar pharmaceuticals [6,7].

Alpert first coined the name HILIC in 1990 [8], although this specific separation mode has been used in polar analyte separations for many years [9–14]. In this chromatographic technique, the polar analytes interact with a hydrophilic stationary phase often *via* hydrogen bonding interactions, dipolar interactions or coulombic interactions. They are eluted with a binary eluent, which consists mainly of a large amount of acetonitrile (usually >60%) and a smaller amount of aqueous solvent. There are several commercially available columns dedicated to HILIC separations [15]. These stationary phases can be classified by their chemistry into several categories, including underivatized silica [16,17], aminopropyl silica [18], amide silica [19], diol silica [20,21], cyclodextrin-bonded silica [14,22], sulfonated polystyrene-divinylbenzene resin [23,24], and sulfoalkylbetaine silica [25]. Nevertheless, it is of great importance to explore new stationary phases in order to expand and hopefully improve this technique.

In this paper, native cyclofrutan 6 (CF6) based stationary phases have been developed as effective stationary phases for the separation of polar analytes in the HILIC mode. Cyclofructans are new types of macrocyclic oligosaccharides [26,27]. They consist of six or more β-(2→1) linked D-fructofuranose units and each unit contains one primary hydroxyl group and two secondary hydroxyl groups, which account for the hydrophilic character of these molecules. Functionalized cyclofructans have proven to be exceptional chiral selectors and broadly useful in enantiomeric separations [28–30]. Although native cyclofructans have very limited capabilities as chiral selectors [28], their unique structure may make it possible for them to be used for achiral separations in the HILIC mode. Among cyclofructans, CF6 has attracted most attention due to its highly defined geometry and its availability in pure form. Therefore, in the present work, CF6 was chosen for development as a new HILIC stationary phase and was compared with three commercial HILIC columns.

* Corresponding author. Tel.: +1 817 272 0632; fax: +1 817 272 0619.
E-mail address: sec4dwa@uta.edu (D.W. Armstrong).

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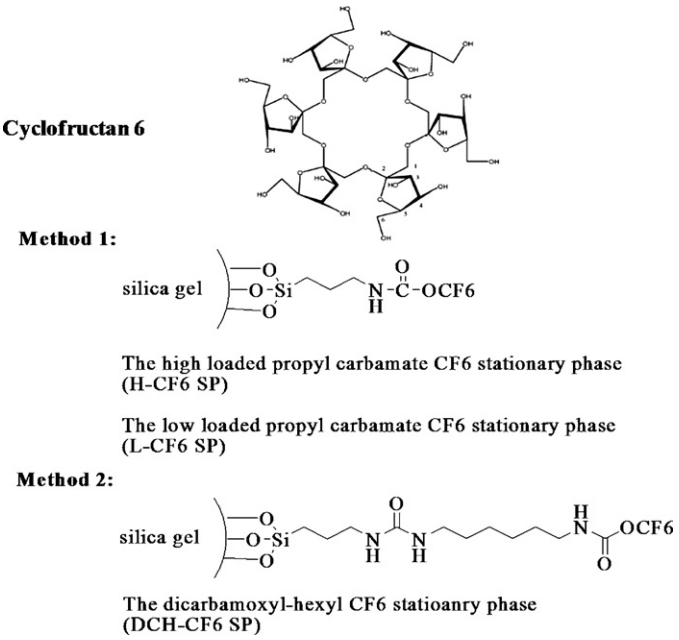

Fig. 1. Structures of cyclofructan 6 bonded stationary phases prepared *via* different methods.

2. Experimental

2.1. Reagents

CF6 was produced *via* inulin fermentation from various microorganisms (for example, *Bacillus circulans* OKUMZ 31B and *B. circulans* MCI-2554) [31–33]. Also, CF6 can be produced by incubation of inulin with the active enzyme cycloinulooligosaccharide fructanotransferase (CFTase) [34]. Consequently, mass produced CF6 could be available at low cost.

Daiso silica of 5 μm spherical diameter with 100 Å pore size and 440 m^2/g surface area was utilized as the supporting material. Anhydrous N,N-dimethylformamide (DMF), anhydrous toluene, anhydrous pyridine, 3-(triethoxysilyl) propylisocyanate, (3-aminopropyl)triethoxysilane, 1,6-diisocyanatohexane, ammonium acetate, acetic acid, and all polar analytes tested in this study were purchased from Sigma–Aldrich (Milwaukee, WI). Acetonitrile of HPLC grade was obtained from EMD (Gibbstown, NJ). Water was purified by a Milli-Q Water Purification System (Millipore, Billerica, MA).

2.2. Synthesis of cyclofructan 6 (CF6) based stationary phase

In this work, native CF6 was chemically bonded to silica gel *via* carbamate linkage by two different methods [28]. Thus far, two stationary phases named the high coverage propyl carbamate CF6 (H-CF6) and the low coverage propyl carbamate CF6 (L-CF6) were produced using the first method (see Section 2.2.1), and one stationary phase named the dicarbamoxyl-hexyl linked CF6 (DCH-CF6) was produced using the second method (see Section 2.2.2). Structures of the newly prepared stationary phases are shown in Fig. 1. Elemental analysis was conducted to confirm the successful synthesis of the CF6 based stationary phases and to determine the cyclofructan coverage. The results are reported in Table 1.

2.2.1. Method 1

A slurry of 3.00 g silica gel in 80 mL of anhydrous toluene was refluxed for 2 h and 10 mL toluene with the residual water was azeotropically removed using a Dean-stark trap. Simultaneously, 3-(triethoxysilyl)propyl isocyanate (0.76 mL, 3.08 mmol for L-CF6; 2.07 mL, 8.00 mmol for H-CF6) dissolved in 15 mL of pyridine was added drop by drop to a solution of CF6 (1.50 g, 1.54 mmol for L-CF6; 2.02 g, 2.08 mmol for H-CF6) in 60 mL anhydrous DMF over 30 min. The reaction was carried out with continuous stirring under a nitrogen atmosphere at 70 °C for 5 h. After cooling to room temperature, the product was mixed with the dried silica gel. This slurry was heated at 110 °C for 12 h to yield the stationary phases. The H-CF6 and the L-CF6 stationary phase had 16.0% and 13.2% carbon loading, respectively.

Table 1
Elemental analysis results of three native CF6 based stationary phases.

	C (%)	H (%)	N (%)
H-CF6	16.0 ± 0.2	2.7 ± 0.1	2.6 ± 0.1
L-CF6	13.2 ± 0.1	2.2 ± 0.1	1.2 ± 0.1
DCH-CF6	14.5 ± 0.1	2.6 ± 0.1	4.3 ± 0.1

2.2.2. Method 2

In this method, 5.00 g silica gel was suspended in 80 mL of anhydrous toluene and refluxed to remove residual water *via* the same approach described in Section 2.2.1. After cooling to room temperature, (3-aminopropyl) triethoxysilane (2.50 mL, 11.00 mmol) was added and the mixture was heated to reflux for 4 h. The reaction was stopped and the modified silica gel was isolated by filtration. After washing with 50 mL portions of toluene, methanol and water, the silica gel was dried at reduced pressure. To a slurry of 3.00 g (3-aminopropyl)-silica gel in 60 mL of dry toluene, 1,6-diisocyanatohexane (2.5 mL, 15.5 mmol) was added with a syringe. The mixture was heated at 70 °C for 2 h. After cooling to room temperature, the liquid phase was removed by suction filtration through an immersion sintered Teflon filter under nitrogen atmosphere. A suspension of cyclofructan 6 in anhydrous DMF (1.45 g, 1.49 mmol) was added to the activated silica and the mixture was heated to 70 °C for 12 h with continuous stirring under a nitrogen atmosphere. After cooling to room temperature, the product (DCH-CF6) was washed with 50 mL portions of DMF, toluene, methanol, water, acetone and dichloromethane, and dried in the vacuum oven, to give 14.5% carbon loading (Table 1).

2.3. HPLC method

The HPLC column packing system is composed of an air driven fluid pump (HASKEL, DSTV-122), an air compressor, a pressure regulator, a low pressure gauge, two high pressure gauges (70 and 40 MPa, respectively), a slurry chamber, check valves, and tubings. The stainless-steel columns (250 mm × 4.6 mm i.d.) were slurry packed with the above-mentioned stationary phases.

All experiments were conducted on Agilent HPLC series 1200 systems (Agilent Technologies, Palo Alto, CA), equipped with a quaternary pump, an autosampler, and a multiwavelength UV–vis detector or a RI detector. For data acquisition and analysis, the Chemstation software version Rev. B.01.03 was used on the system in Microsoft Windows XP environment. The injection volume was 5 μL and the flow rate of the mobile phase was 1.0 mL/min. Separations were carried out at room temperature if not otherwise specified. Each sample was analyzed in duplicate. The separations of standard mixtures were carried out in the HILIC mode with a mobile phase composed of 60–95% acetonitrile in 20 mM ammonium acetate, pH = 4.1. The ammonium acetate buffer concentration and pH were varied between 0 and 20 mM and pH = 3.0–6.5, respectively, as a part of the optimization process. The 20 mM buffer solution was prepared by dissolving 1.54 g ammonium acetate in 200 mL of purified water. The stock solution was transferred to a 1000 mL volumetric flask which was filled to just

Table 2
Physical evaluation of the six tested columns.

	H-CF6	L-CF6	DCH-CF6	Astec Diol	Astec Cyclobond I 2000	ZIC-HILIC
k (uracil)	1.10	1.01	0.63	0.45	0.69	0.72
N/m	61 600	90 000	50 400	58 800	82 800	54 400
Peak symmetry	0.86	0.70	0.63	0.97	1.39	0.75
k (cytosine)	5.94	5.90	2.13	2.47	3.22	3.21
N/m	43 700	64 800	19 600	55 600	63 800	37 700
Peak symmetry	0.77	0.69	0.52	0.91	1.43	0.75

Uracil and cytosine were tested as standards under the following conditions: acetonitrile/20 mM ammonium acetate, pH = 4.1, 90/10 (v/v); column temperature: 20 °C; flow rate: 1.0 mL/min; UV detection: 254 nm.

below the mark with purified water. Then the desired pH can be adjusted by using 99.7% acetic acid. Appropriate amounts of acetonitrile and the buffer solution were mixed thoroughly and degassed by ultrasonication under vacuum for 5 min before use.

Several types of polar compounds were used to investigate and evaluate the separation properties of the newly prepared stationary phases in the HILIC mode (see Fig. 2). Nucleic acid bases and nucleosides are polar compounds of significant biological and pharmaceutical interests. Uracil is included as it is often employed as a void volume marker in RPLC. Xanthines, β-blockers, salicylic acid and its derivatives, and maltooligosaccharides also were used to examine the potential of the cyclofructan based columns in the HILIC mode and to access the retention mechanism. After more than 1000 injections, the column still gave good selectivity and similar retention for the same analytes. There was no significant deterioration observed for the column after six months of use. It is indicated that these CF6 columns are stable, have good efficiencies and good reproducibility.

Three commercial columns were chosen for comparison, on the basis of differences in their structures, their popularity, and availability of published data on separating polar analytes. The ZIC-HILIC, 250 mm × 4.6 mm, 5 μm and 200 Å, was purchased from Merck SeQuant (Darmstadt, Germany). This column is functionalized with a sulfopropylbetaine ligand that overall confers a high capability of binding water and various coulombic interactions with a low contribution of hydrogen bonding interactions [25]. These unique properties play an important role in the HILIC mode separations. Astec Diol HPLC column and Astec Cyclobond I 2000 column, 250 mm × 4.6 mm, 5 μm and 100 Å, were obtained from Supelco (Bellefonte, PA). Both the Astec Diol HPLC and Astec Cyclobond I 2000 columns have hydroxyl groups as active functionalities.

For the calculation of chromatographic data, t_0 was determined by the refractive index change caused by the sample solvent or by injecting toluene in the HILIC mode. Column efficiency was evaluated using uracil and cytosine as the test compounds. The optimized mobile phase consisted of 90/10 (v/v) acetonitrile with 20 mM ammonium acetate buffer pH = 4.1.

Thermodynamic data were obtained using isothermal conditions over a temperature range of 20–70 °C at 10 °C intervals, with a mobile phase containing acetonitrile and 20 mM ammonium acetate, pH = 4.1 (90/10, v/v). The precision of the controlled temperature was ±0.1 °C.

3. Results and discussion

The elemental analysis data in Table 1 indicate that the H-CF6 stationary phase has a higher native CF6 loading than does the L-CF6 stationary phase. Table 1 also shows that the carbon loading of DCH-CF6 stationary phase is 14.5%. However, this does not mean that the coverage of CF6 on this column was as high as it was on the L-CF6 stationary phase. The carbon loading value of the DCH-CF6 stationary phase also reflects the high amount of C_6-aliphatic covalent spacer present. The column efficiency data in Table 2 show that some of the synthesized stationary phases are competitive with or superior to current commercial stationary phases. The compounds used to measure column efficiency were selected because they are used to measure the column efficiency of the commercial ZIC-HILIC column. The L-CF6 column had (90,000 plates m^{-1} for uracil and 65,000 plates m^{-1} for cytosine) the highest peak efficiency among six tested columns, when using a mobile phase containing acetonitrile and 20 mM ammonium acetate, pH = 4.1 (90/10, v/v). The H-CF6 column also shows good peak symmetry when separating the same analytes with the same mobile phase. However, the DCH-CF6 column had poorer efficiencies and peak shapes.

3.1. Optimized separation of polar mixtures

Initially, the characterization of these HILIC stationary phases was done by using test mixtures of nucleic acid bases and nucleosides, β-blockers, xanthines, salicylic acid and its analogues, and maltooligosaccharides. The structures of these compounds are shown in Fig. 2.

Figs. 3–7 compare the separation performance of the three newly prepared cyclofructan 6 based stationary phases with three commercially available columns.

Fig. 3 shows chromatograms of nucleic acid bases and nucleosides obtained on the six columns under the same mobile phase conditions with acetonitrile and 20 mM ammonium acetate, pH = 4.1 (90/10, v/v). These 10 analytes have a "separation window" of around 40 min on the H-CF6 column, of around 30 min on the L-CF6 and ZIC-HILIC columns, and of less than 20 min on the other columns. Comparable elution orders were observed on all six columns. The H-CF6 column and the ZIC-HILIC column provided the best performance in separating nucleic acid bases and nucleosides. The selectivity factors ($\alpha > 1.07$) and resolutions ($Rs > 1.4$) of all adjacent peaks were observed for all ten nucleic acid compounds on the H-CF6 column. Except for thymidine and uracil ($\alpha = 1.08$; $Rs = 1.3$) and adenine and adenosine ($\alpha = 1.09$; $Rs = 1.3$), the other adjacent peaks were baseline separated on the ZIC-HILIC column. The L-CF6 column did not perform as well as the former columns, in terms of selectivity and resolution. For example, the pairs adenine and adenosine ($\alpha = 1.04$; $Rs = 1.0$), cytosine and guanine ($\alpha = 1.02$; $Rs = 0.7$), cytidine and guanosine ($\alpha = 1.03$; $Rs = 0.8$) were partially separated on this column. It is believed that the different coverage of cyclofructan is the main reason for the reversed elution order of adenine and adenosine, and the different resolutions for cytosine, guanine, cytidine and guanosine observed on the H-CF6 and the L-CF6 columns. Both the steric interactions and number of available silanol groups would be somewhat different for these closely related stationary phases. The elution profile on the DCH-CF6 column showed that there was no selectivity between thymine and uracil and there were three partial separations – uracil and thymidine, adenosine and adenine, cytosine and guanine. Three partially separated pairs were obtained on the Astec Cyclobond I 2000 column and there were four partial separations on the Astec Diol HPLC column. There are previous reports on the HILIC separation of fewer

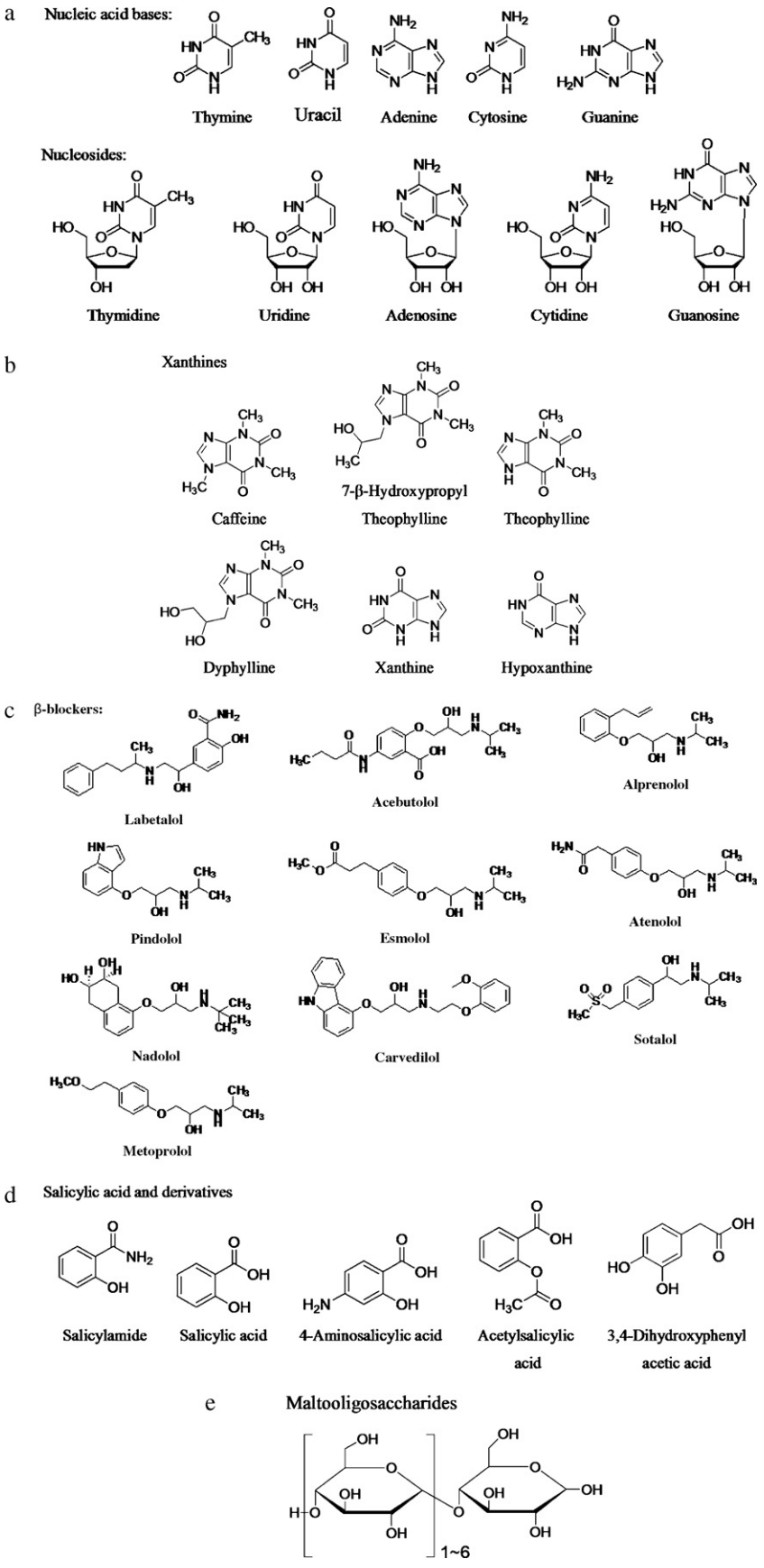

Fig. 2. Test compounds for chromatographic characterization of the columns in the HILIC mode. (a) Nucleic acid bases and nucleosides; (b) xanthines; (c) β-blockers; (d) salicylic acid and derivatives and (e) maltooligosaccharides.

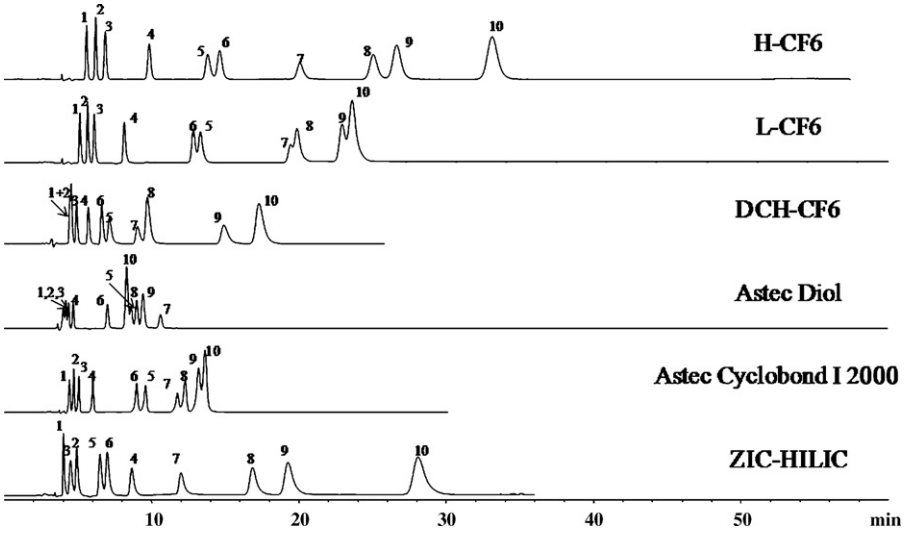

Fig. 3. Separation of nucleic acid bases and nucleosides on the six compared columns. Mobile phase: acetonitrile/20 mM ammonium acetate, pH = 4.1, 90/10 (v/v); flow rate: 1.0 mL/min; UV detection: 254 nm. Compounds: (**1**) thymine; (**2**) uracil; (**3**) thymidine; (**4**) uridine; (**5**) adenine; (**6**) adenosine; (**7**) cytosine; (**8**) guanine; (**9**) cytidine and (**10**) guanosine.

nucleic acid bases and nucleosides than reported here [7,17,35–38]. In these cases, overlapping analytes were replaced with unrelated amines, such as theophylline and caffeine. Also thermal or solvent gradient conditions were used. No other isocratic separations of the ten analytes in this study (Fig. 3) have been reported.

All xanthines were baseline separated on the H-CF6, the L-CF6 and the Astec Cyclobond I 2000 columns, as shown in Fig. 4. The separation times were less than 15 min for all columns. Under the same mobile phase condition, the DCH-CF6 and the ZIC-HILIC columns were less successful in separating these types of analytes, since two pairs of partially separated peaks were observed on the DCH-CF6 and three on the ZIC-HILIC. Previously, it was proposed that the poor retention of xanthines on the ZIC-HILIC column could be due to the weaker ionic-dipolar interactions or coulombic interactions between these analytes and the stationary phases as xanthines remain neutral in a hydroorganic environment [39]. Thus, differences in the hydrophilic interactions between these analytes and the stationary phases are not significant. The Astec Diol HPLC column also is a poor choice for this separation given the three coeluting peaks obtained. Additionally, compared with other columns, the H-CF6 column consistently produced higher retention factor values. This result can be related to a greater number of available hydroxyl groups that can enhance hydrogen bonding interactions between the polar solutes and the stationary phase.

In the case of the β-blockers, the mobile phase composition was adjusted in order to separate as many analytes as possible. The resolution between adjacent analytes improved with increasing concentrations of acetonitrile. When the mobile phase contained percentages higher than 90% of acetonitrile, nadolol and atenolol did not elute. Fig. 5 shows the performance of the HILIC columns for the separation of β-blockers. Among the six tested columns, the L-CF6 was the most effective and retentive column for separating this mixture of β-blockers. All ten compounds were almost baseline separated. There were two partially separated pairs: alprenolol and esmolol ($\alpha = 1.05$, $Rs = 1.3$) and labetalol and pindolol ($\alpha = 1.04$, $Rs = 1.1$) on this column. The Astec Diol HPLC column was the second most effective in terms of selectivity and resolution with eight analytes baseline separated in 42 min. Pindolol and esmolol were partially separated with $\alpha = 1.02$, $Rs = 0.5$, and metoprolol and sotalol were partially separated ($\alpha = 1.04$, $Rs = 1.0$). However, the H-CF6 and Astec Cyclobond I 2000 columns did not perform as well in separating this mixture of analytes with shorter retention and worse selectivitities. For example, labetalol, pindolol and metoprolol coeluted on the H-CF6 column, as did sotalol and acebutolol. Labetalol and pindolol were partially separated on the Astec

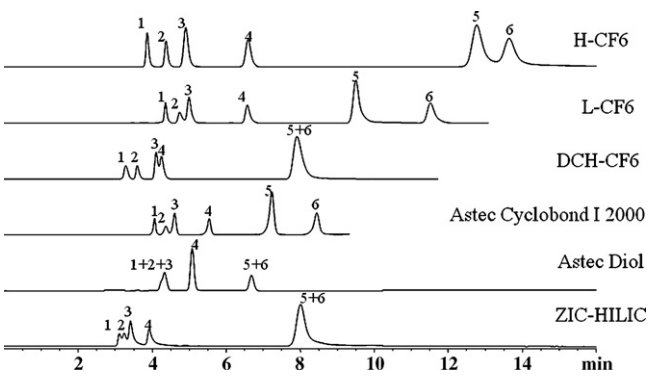

Fig. 4. . Separation of xanthines on the six compared stationary phases. Mobile phase: acetonitrile/20 mM ammonium acetate buffer, pH = 4.1, 90/10 (v/v); flow rate: 1.0 mL/min; UV detection: 254 nm. Compounds: (**1**) caffiene; (**2**) 7-β-hydroxypropyl theophylline; (**3**) theophylline; (**4**) dyphilline; (**5**) hypoxanthine and (**6**) xanthine.

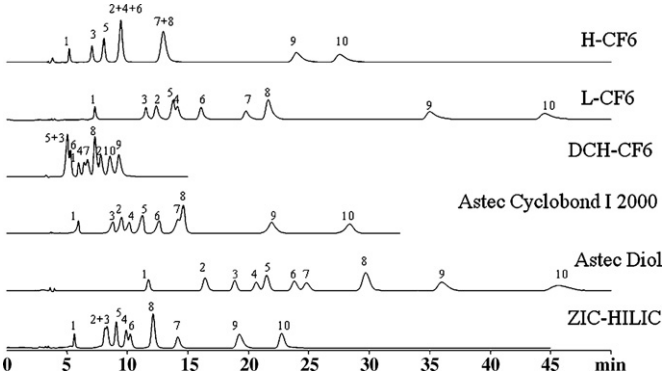

Fig. 5. Separation of β-blockers on the six compared stationary phases. Mobile phase: acetonitrile/20 mM ammonium acetate buffer, pH = 4.1, 90/10 (v/v); flow rate: 1.0 mL/min; UV detection: 254 nm. Compounds: (**1**) carvedilol; (**2**) labetalol; (**3**) alprenolol; (**4**) pindolol; (**5**) esmolol; (**6**) metoprolol; (**7**) sotalol; (**8**) acebutolol; (**9**) nadolol and (**10**) atenolol.

Cyclobond I 2000 column and sotalol and acebutolol coeluted. Neither the DCH-CF6 column nor the ZIC-HILIC column was adequate for the separation of such mixtures even under optimum conditions. Longer retention and better selectivities of β-blockers were obtained on the L-CF6 as compared to the H-CF6 column. This likely results from the mixed-mode interactions of the analytes with the stationary phase (hydrogen bonding interaction and silanol group adsorption) [40].

As shown in Fig. 6, the H-CF6, the L-CF6 and the ZIC-HILIC columns can separate salicylic acid and its derivatives with good peak shapes and efficiencies. Comparing the retention and selectivities of these acidic analytes on the cyclofructan-based stationary phases, the H-CF6 produced greater retention (around 9 min) for salicylic acid and its analogues than the L-CF6 column (around 7 min). This could be attributed to the higher cyclofructan content of the stationary phase. In comparison, the H-CF6 and ZIC-HILIC columns show similar retentions and selectivities for the first four eluted analytes but the latter column has greater retention (around 11 min) and a more tailing peak for 3,4-dihydroxyphenylacetic acid than does the H-CF6 column. Co-elution or partial separations occurred on the DCH-CF6, the Astec Cyclobond I 2000 and the Astec Diol HPLC columns. Comparing previous reports [17,41] on the separation of salicylic acid and its analogues on commercial columns, under similar mobile phase conditions, the performance of the H-CF6 column is better than that of the HILIC silica (Agilent) column, the ZIC-HILIC column and the YMC Pack NH_2 column, but comparable to that of the TSK-gel Amide-80 column and the polyhydroxyethyl A column.

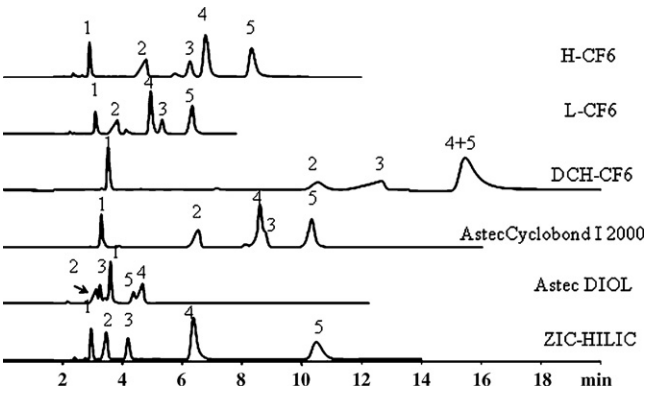

Fig. 6. Separation of salicylic acid and its derivatives on the six compared stationary phases. Mobile phase: acetonitrile/20 mM ammonium acetate buffer, pH = 4.1, 85/15 (v/v); flow rate: 1.0 mL/min; UV detection at 254 nm. Compounds: (**1**) salicylamide; (**2**) salicylic acid; (**3**) 4-aminosalicylic acid; (**4**) acetylsalicylic acid and (**5**) 3,4-dihydroxyphenylacetic acid.

Native cyclodextrin-based columns are particularly useful for analysis of oligosaccharides due to possible hydrogen bonding interactions of the oligosaccharide hydroxyl groups with those of the cyclodextrin stationary phase [20,22,42,43] in a mobile phase consisting of acetonitrile/water 65/35 (v/v). CF6-based columns have somewhat analogous hydrogen bonding capabilities but a different geometry. The separation of maltooligosaccharides (with two to seven glucose units) was conducted on the six columns

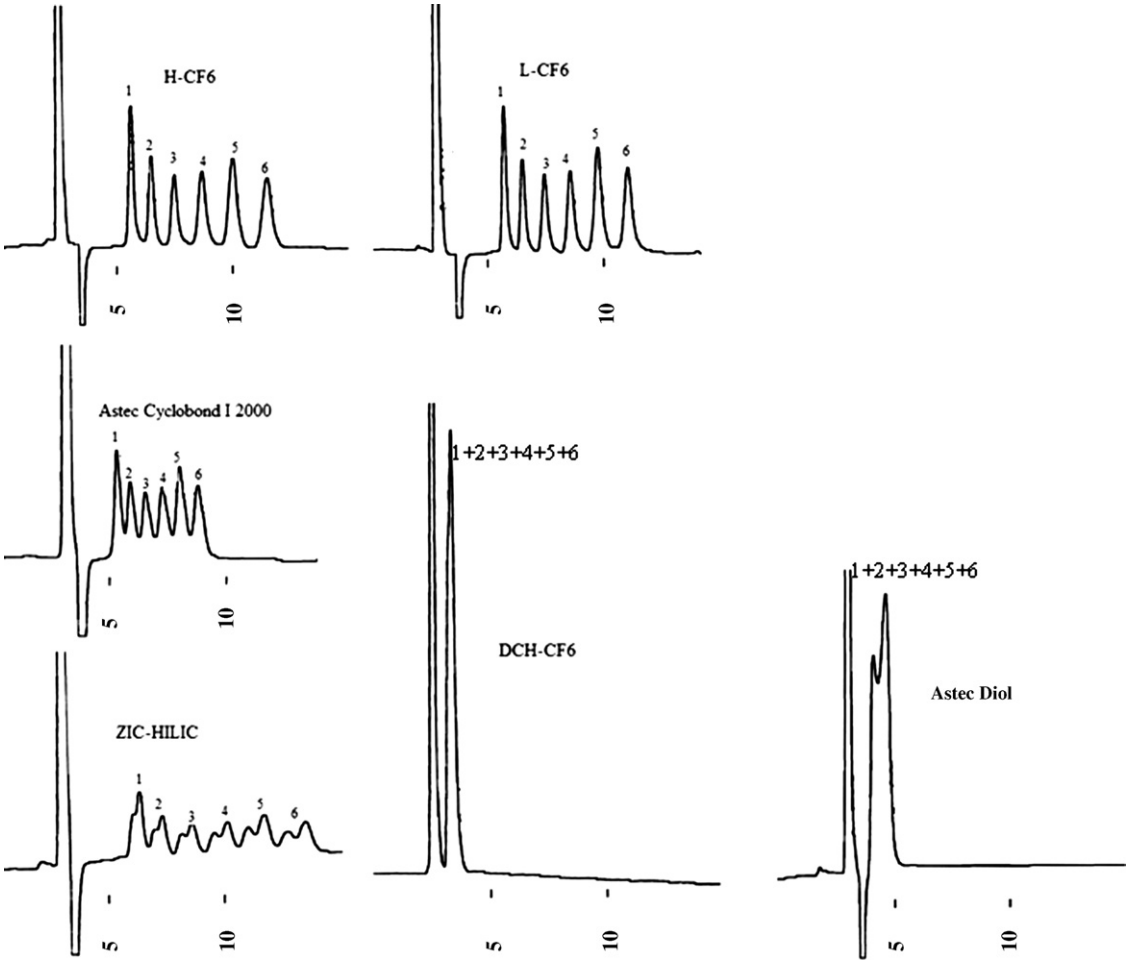

Fig. 7. Separation of maltooligosaccharides on the six compared columns. Mobile phase: acetonitrile/water 65/35 (v/v); flow rate: 1.0 mL/min; RI detection. Compounds: (**1**) maltose; (**2**) maltotriose; (**3**) maltotetraose; (**4**) maltopentaose; (**5**) maltohexaose and (**6**) maltoheptaose.

as shown in Fig. 7. In this separation environment, the retention times predictably increased with the number of analyte hydroxyl groups, which corresponds to the degree of polymerization (dp). Hence, maltoheptaose (dp = 7) tends to be retained more than maltohexaose (dp = 6), and maltose (dp = 2) is always eluted first. With a mobile phase of acetonitrile and water (65/35, v/v), baseline separation of all maltooligosaccharides was achieved on both the H-CF6 and the L-CF6 columns but the analytes were more retained on the former column (separation times around 12 min on the H-CF6 column and 11 min on the L-CF6 column). The Cyclobond I 2000 column showed a moderate capability in separating these oligomers with this mobile phase in terms of shorter retentions (running time is around 9 min) and slightly poorer selectivities. Under the same mobile phase condition, peak splitting of all sugars was observed on the ZIC-HILIC column as the anomeric separation occurred. There was no separation of maltooligosaccharides on the DCH-CF6 column and on the Astec Diol HPLC column. For native saccharides, anomeric separations have been probed on the cyclofructan-based column when acetonitrile content in the mobile phase exceeded 80%. Similarly, anomeric separations have been reported on cyclodextrin-bonded columns in acetonitrile rich mobile phases [22,44,45]. Clearly, the CF6-based stationary phases are exceptional for sugar/carbohydrate separations in the HILIC mode and do not react with reducing saccharides. Consequently, they are good for quantitative analysis of carbohydrates.

The H-CF6 column produced the best separation for the nucleic acid bases and nucleosides, xanthine analogues, and also afforded better separation of maltooligosaccharides. A better separation of β-blockers was achieved on the L-CF6 column. The H-CF6, ZIC-HILIC, and L-CF6 all separated the polar acidic analytes effectively. The main difference between the H-CF6 and L-CF6 stationary phases is the cyclofructan content on the silica support. The difference in retention and selectivity observed on both columns depends not only on the degree of cyclofructan coverage and binding chemistry but also on the nature of the analyte and mobile phase composition.

3.2. Impact of mobile phase variables on retention and selectivity

Experimental parameters were selected to study their effects on retention and selectivity. These included the nature and amount of organic modifier, salt concentration and buffer pH, and temperature.

3.2.1. Nature and amount of organic modifier

In the HILIC mode, acetonitrile is most commonly used as the organic modifier. The optimized separations for polar mixtures (Fig. 2) were achieved with a large amount of acetonitrile and a smaller amount of the aqueous part of the mobile phase. Acetonitrile is advantageous for the HILIC mode, because it is a poor hydrogen bonding solvent and it is polar and miscible with water in all proportions. Thus, it provides an environment for attractive and discriminative interactions of polar solutes with the hydrophilic stationary phases. Usually, protic solvent, such as methanol, affords insufficient retention and incomplete separation for polar analytes. At higher percentages of acetonitrile in the mobile phase, the retention factor is governed by a contribution of hydrogen bonding, dipolar and hydrophilic interactions between the solute and the stationary phase. The solutes with a higher number of available polar sites, such as the ribose structural element in nucleosides (which is absent in nucleic acid bases) have stronger interactions with polar stationary phases.

Fig. 8 shows a typical dependence of retention factors (k) on acetonitrile content in the mobile phase for five nucleic acid bases and five nucleosides on the H-CF6 column. The retention factor profiles of all analytes are similar in shape. When acetonitrile in the mobile phase is in the range of 20–60%, retention is minimal as all solutes elute near the dead volume. Decreasing the acetonitrile content in the mobile phase (<20%) results in longer retention, which is similar to what is found in RPLC. However, this trend is not substantial, as the cyclofructan-based stationary phase has very high hydrophilic nature [8,39,46]. The HILIC separation environment becomes predominant when the acetonitrile amount exceeds ~60% by volume. Then the retention factors increase is steep for the hydrogen-bonding interaction domain. However, all analytes produced broad peaks or were not eluted if the acetonitrile amount was higher than 95%, often due to the solubility limitations of these compounds. Similar dependences were obtained on the other tested columns.

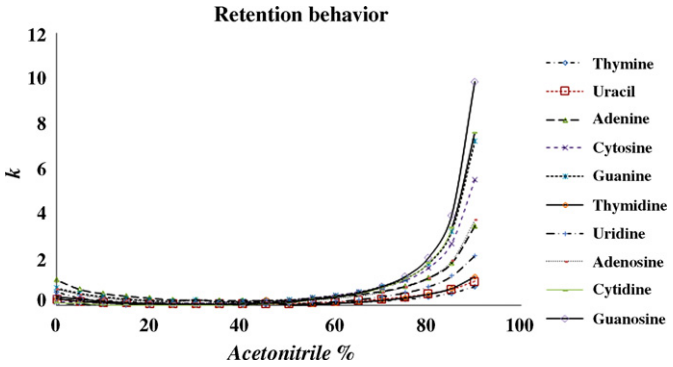

Fig. 8. Effect of acetonitrile content on the retention (k) of ten tested nucleic acid bases and nucleosides on the H-CF6 column. Mobile phase: acetonitrile and 20 mM ammonium acetate, pH = 4.1. Flow rate: 1.0 mL/min. UV detection at 254 nm.

3.2.2. Buffer effects

3.2.2.1. Salt concentration. Various salts (e.g. triethylammonium phosphate, or carbonate salts) typically used in RPLC are not suitable for HILIC, as they are poorly soluble in mobile phases containing high amounts of acetonitrile [17]. Initially, triethylammonium acetate and ammonium acetate were used for HILIC separations. In most cases, these columns have better performance when using ammonium acetate buffer. Many researchers also used ammonium formate for separation because they could achieve higher selectivity and better compatibility with MS detection [36]. The effect of the amount of ammonium acetate in the mobile phase also was evaluated. The investigation was carried out by varying the salt concentration of the buffer in the range of 0–20 mM at pH = 4.1 (acetonitrile percentage was kept constant). Generally, the separation of nonionic solutes, such as maltooligosaccharides, did not show significant differences either in terms of retention or selectivity, when changing the salt concentration from 0 mM to 20 mM. For nucleic acid compounds and xanthines, slight differences in the separations were observed when the salt concentration changed. However, there was a substantial influence of ionic strength on β-blockers and salicylic acid analogues, not only on their retention but also on resolution. The obtained data indicated that the retention of β-blockers increased if no buffer was used. The data in Table 3 show retention parameters for nadolol and atenolol, as representative β-blockers, at two ammonium acetate concentrations (5 mM and 20 mM, at the same pH) on different HILIC columns. The results indicate that the resolutions increased greatly when the buffer concentration increased from 5 mM to 20 mM but the selectivity changed only slightly. Similar results were observed with salicylic acid and analogues.

3.2.2.2. Buffer pH. Buffer pH influences the retention behavior of some solutes, since it affects the ionization of solutes and/or sta-

Table 3
Effect of buffer concentration on retention parameters of nadolol (k_1) and atenolol (k_2). Mobile phase: acetonitrile/ammonium acetate, pH = 4.1, 90/10 (v/v); flow rate: 1.0 mL/min; UV detection at 254 nm.

Column	5mM ammonium acetate, pH = 4.1				20mM ammonium acetate, pH = 4.1			
	k_1	k_2	α	Rs	k_1	k_2	α	Rs
H-CF6	11.24	13.00	1.14	2.28	7.87	9.16	1.15	2.63
L-CF6	19.32	24.48	1.25	4.88	12.28	15.77	1.26	6.16
DCH-CF6	1.00	1.15	1.08	1.00	1.79	2.05	1.09	1.54
ZIC-HILIC	13.95	16.05	1.14	2.79	6.32	7.61	1.18	3.24
Astec Cyclond I 2000	5.95	8.02	1.30	4.10	6.88	9.44	1.33	6.65
Astec Diol	19.05	23.01	1.20	2.86	10.48	13.31	1.25	6.06

Mobile phase: acetonitrile/ammonium acetate, pH = 4.1, 90/10 (v/v); flow rate: 1.0 mL/min; UV detection: 254nm.

tionary phase functional groups. This investigation was carried out by varying the buffer pH from 3.0 to 6.5 at a fixed buffer concentration and acetonitrile content in the mobile phase. For nucleic acid bases, nucleosides, xanthines, only slight retention variations with pH differences were observed. This is consistent with the general observations for HILIC-type commercial columns reported earlier [35,37,47]. Thus, little or no pH effect on retention of nonionic solutes, such as maltooligosaccharides, can be expected. On the other hand, the retention of β-blockers is greatly affected by buffer pH. Nadolol and atenolol were again taken as representative examples to show the effect of buffer pH on the retention in Fig. 9(a) and (b) with a mobile phase of acetonitrile/20 mM ammonium acetate 90/10 (v/v). The retention factors of nadolol and atenolol increased with the pH on all columns except for the DCH-CF6. The sharpest increase was observed in the range of 4.0–5.0, which correspond to the pK_bs of these compounds (nadolol, pK_b = 4.3; atenolol pK_b = 4.4) [48]. The highest retention resulted from two columns, i.e., the L-CF6 and Astec Diol. However, an increase in retention does not necessarily translate into improved selectivity, as the selectivity on all the tested columns for these analytes was the highest at a pH of approximately 4 (Fig. 9(c)). The maximum resolution also was observed around pH = 4 as shown in Fig. 9(d). Both the selectivity and resolution of β-blockers were best on the L-CF6 stationary phase. For salicylic acid and analogues compounds, the buffer pH only appreciably affected the retention of 3,4-dihydroxyphenylacetic acid.

While the nature and amount of organic modifier in the mobile phase affect the retention and separation of all analytes, the buffer concentration and pH influence some but not others. There was a significant impact of buffer concentration and buffer pH on the retention and resolution of β-blockers but a minor effect on the retention parameters of nucleic acid bases, nucleosides, xanthines, and maltooligosaccharides. As expected, higher buffer concentrations increased the eluting strength of the mobile phase, thus resulting in less retention of anionic components, but it did not

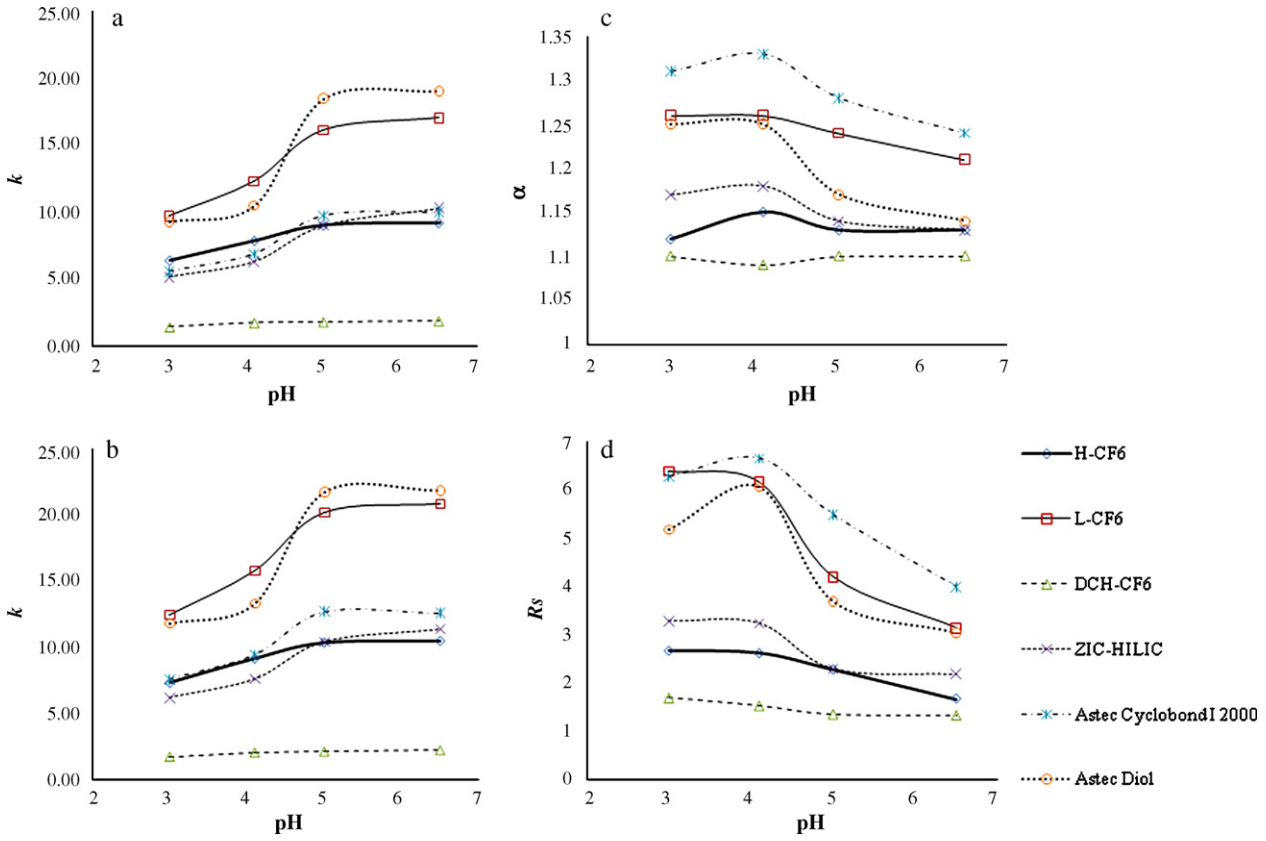

Fig. 9. Effect of buffer pH on the retention, k, of nadolol (a) and atenolol (b) and on the selectivity, α, (c) and resolution, Rs, (d) of nadolol and atenolol on the six compared stationary phases. Mobile phase: acetonitrile/20 mM ammonium acetate, 90/10 (v/v); flow rate: 1.0 mL/min; UV detection at 254 nm.

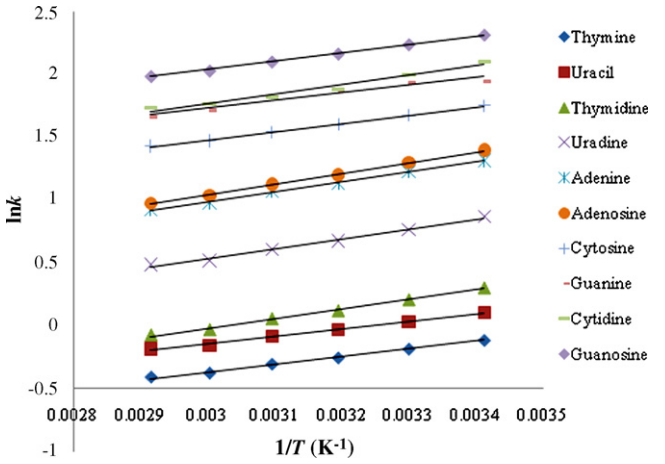

Fig. 10. Dependences of logarithms of retention factors (ln k) on the inverse of temperature ($1/T$) for nucleic acid bases and nucleosides using the H-CF6 column. Mobile phase: acetonitrile/20 mM ammonium acetate, pH = 4.1, 90/10 (v/v); flow rate: 1.0 mL/min; UV detection at 254 nm.

affect the retention of neutral compounds. Buffer pH effects on retention and resolution are directly related to nature of analytes (e.g., pK_a values, solubilities, etc.).

3.2.3. Thermodynamic study

There have been numerous studies of temperature effects on solute retention in the HILIC mode [17,35,38]. The dependence of the natural logarithms of retention factors ($\ln k_i$) on the inverse of temperature ($1/T$) is routinely used to determine thermodynamic data which can relate to the separation mechanism.

It is generally accepted that the HILIC mode separation is based on the formation of reversible associates (hydrogen bonding associates for polyhydroxyl stationary phases, coulombic interactions for the zwitterionic stationary phases, e.g., the ZIC-HILIC column) that are created by intermolecular interactions of polar analytes and hydrophilic stationary phase. In this work, thermodynamic data (ΔG_i, ΔH_i, ΔS_i) were calculated according to the Gibbs–Helmholtz equation:

$$\Delta G_i = \Delta H_i - T\Delta S_i = -RT \ln K_i \quad (1)$$

where ΔG_i is the molar Gibbs energy, ΔH_i is the molar enthalpy, ΔS_i is the molar entropy, K_i is the solute partition coefficient, R is the universal gas constant, and T is the temperature (K).

The dependence of analyte retention on the temperature can be expressed by the van't Hoff equation:

$$\ln k_i = \frac{\Delta H_i}{RT} + \frac{\Delta S_i}{R} + \ln \phi \quad (2)$$

where k_i is the retention factor of a solute, ΔH_i is the molar enthalpy of transfer of a solute in the chromatographic system, ΔS_i is the molar entropy, and Φ is the phase ratio of the chromatographic column ($\Phi = V_M/V_S$).

The van't Hoff plots (dependence of $\ln k_i$ on $1/T$) were linear within the studied temperature interval for nucleic acid bases and nucleosides using the H-CF6 column, as shown in Fig. 10. Similar dependencies were obtained for other analytes. Linear dependencies indicate no change of interaction mechanism within the measured temperature range. Table 4 lists the thermodynamic data for nucleic acid bases and nucleosides on the tested columns. The ΔH_i values calculated from the slope of the plot of Eq. (2) were negative for all analytes on the H-CF6 column, ranging from −4.94 to −7.08 kJ/mol. The negative sign of ΔH_i indicates an exothermic process. The ΔH_i values obtained on the L-CF6 column varied from

Table 4
The thermodynamic parameters resulting from linear regression (ln k *vs.* $1/T$) for nucleic acid bases and nucleosides on the HILIC columns. Mobile phase: acetonitrile/20 mM ammonium acetate, pH = 4.1, 90/10 (v/v); flow rate: 1.0 mL/min; UV detection: 254 nm.

Analyte	H-CF6			L-CF6			ZIC-HILIC			Astec Cyclobond I 2000		
	ΔH (kJ/mol)	$\Delta S'^a$ (J/mol K)	Correlation coefficient	ΔH (kJ/mol)	$\Delta S'^a$ (J/mol K)	Correlation coefficient	ΔH (kJ/mol)	$\Delta S'^a$ (J/mol K)	Correlation coefficient	ΔH (kJ/mol)	$\Delta S'^a$ (J/mol K)	Correlation coefficient
Thymine	−5.09	−11.37	0.9953	−4.07	−8.56	0.9977	−4.06	−14.12	0.9968	−5.05	−14.72	0.9922
Uracil	−4.94	−9.08	0.9944	−4.09	−6.90	0.9979	−2.21	−3.24	0.9918	−5.48	−14.54	0.9879
Thymidine	−6.37	−12.41	0.9957	−5.21	−9.31	0.9699	−3.52	−9.87	0.9971	−6.94	−17.91	0.9855
Uradine	−6.54	−8.22	0.9908	−5.75	−7.24	0.9986	−2.23	5.10	0.9983	−7.35	0.34	0.9857
Adenine	−6.57	−4.56	0.9987	−5.49	−0.63	0.9989	−2.92	−0.83	0.9971	−6.05	−5.90	0.9925
Adenosine	−7.08	−5.64	0.9982	−5.87	−2.29	0.9991	−2.30	2.17	0.9996	−7.09	−10.04	0.9903
Cytosine	−5.44	2.91	0.9976	−4.65	5.91	0.9987	−3.05	6.21	0.9957	−5.07	−0.42	0.9937
Guanine	−5.10	6.02	0.9454	−6.44	0.71	0.8439	0.97	22.95	0.5749	−5.21	−0.48	0.9964
Cytidine	−6.26	2.85	0.9747	−6.68	0.64	0.9993	−2.25	13.60	0.9445	−7.97	−8.69	0.9934
Guanosine	−5.48	7.42	0.9984	−5.10	6.05	0.9997	2.71	33.51	0.9956	−6.64	−4.10	0.9949

[a] $\Delta S'$ was calculated from the plot intercept (Eq. (2)). $\Phi = V_M/V_S$, V_M was obtained from the flow rate and dead time t_0, assuming negligible extra-column volume. V_S was obtained by the geometric internal volume of the column minus V_M. Here, V_S is the total stationary volume, including the volume of bonded stationary phase and the volume of the supporting material. Since the V_S is the total volume of stationary phase rather than the volume of the absorbing surface layer, we use the prime symbol ($\Delta S'$).

−4.07 to −6.68 kJ/mol. For the Astec Cyclobond I 2000 column, the ΔH_i values ranged from −5.05 to −7.97 kJ/mol with high linearity. Comparing with the thermodynamic data obtained from other columns, the ΔH_i values from the ZIC-HILIC were relatively greater, ranging from 2.71 to −4.06 kJ/mol. Additionally, there were two positive ΔH_i values calculated from the plots on the ZIC-HILIC column, including guanine (0.97 kJ/mol) and guanosine (2.71 kJ/mol). The $\Delta S_i'$ values on the ZIC-HILIC were more positive than the values obtained from all other columns. It was evident that entropic contributions are more dominant on the ZIC-HILIC stationary phase. Guanine showed a nonlinear van't Hoff plot on the ZIC-HILIC that was previously reported by Marrubini et al. [35]. It suggested that guanine's retention was influenced by mixed retention mechanism, ion-exchange and displacement of waters of hydration on the stationary phase. In most other cases, correlation coefficients are greater than 0.99, which indicates that one retention mechanism dominates [49]. For the polyhydroxyl functionalized stationary phases, hydrogen bonding interactions between the polar solute and the hydrophilic stationary phases are dominant and enthalpic in nature. For the ZIC-HILIC column, the influence of coulombic interaction must be taken into consideration.

4. Conclusions

The retention behavior afforded by HILIC offers the potential for dramatic changes in selectivity compared to RPLC. The native CF6 based stationary phases have been successfully synthesized and evaluated. It appears that these new stationary phases have advantages over popular commercial columns in separating nucleic acid bases, nucleosides, xanthines, β-blockers and salicylic acid and its derivatives, and carbohydrates in the HILIC mode. Their performance in the HILIC mode can provide increased retention, improved selectivity and resolution for compounds, which are difficult to retain and separate in RP-HPLC. Additionally, CF6 columns are stable, have good efficiency, good reproducibility, and do not react with reducing sugars/carbohydrates. Consequently, they seem to be good for quantitative analysis. More detailed evaluations are currently underway and derivatization of the CF6 may further broaden its application.

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