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Screening strategies for achiral supercritical fluid chromatography employing hydrophilic interaction liquid chromatography-like parameters

M. Ashraf-Khorassani [a], L.T. Taylor [a,*], E. Seest [b]

[a] Department of Chemistry, Virginia Tech, Blacksburg, VA 24061, USA
[b] Eli Lilly Co., Indianapolis, IN 46285, USA

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ABSTRACT

A thorough evaluation of 5 μm bare silica from two major vendors for achiral supercritical fluid chromatography of polar analytes has been carried out. Columns were the same dimension, and a virgin column was reserved for each modifier-mixture combination. Three mixtures were prepared and chromatographically separated via a gradient of methanol-modified CO_2 that incorporated 5% (w/w) water as a neutral additive. Mixture (A) invoked both trifluoroacetic acid and water as additives. Mixture (B) utilized isopropyl amine and water; while mixture (C) employed either ammonium acetate and water as additives or only water. Regardless of the mixture components and mobile phase composition, duplicate separations with superior selectivity and excellent peak resolution were observed on five analysis days over a 15-day period. Subsequent removal of water (i.e. primary additive) from each of the mobile phases led to lower selectivity for early eluting components but excellent peak resolution prevailed for later eluting peaks during a later 5-day testing period with only the secondary additive. The re-introduction of 5% water into the mobile phase (after allowing the bare silica columns that were used with no water to sit for 30 days) slowly yielded the original separation after approximately five injections. A hydrophilic interaction liquid chromatography (HILIC)-like mechanism for SFC whereby analyte partitions between water absorbed on the silica and water in the mobile phase is proposed. The general utility of this experimental approach with bare silica was subsequently demonstrated by single injection of ten drug-like compounds with each of the four mobile phases that previously were utilized with the three model compound mixtures. In each case, sharp peaks were observed for each drug-like compound regardless of the additive although retention times varied with the additive employed.

1. Introduction

The general perception of supercritical fluid chromatography (SFC) has changed over the years since it was first introduced in the late 1960s. Packed columns (pc) were popular for a while, but the period 1980–1995 saw tremendous interest in the application of wall coated open tubular columns for SFC. During this time, SFC was thought to be applicable to only nonpolar and intermediate polarity analytes whereas separation of polar analytes was primarily reserved for normal phase HPLC. If pcSFC was performed in those days, the mobile phase was highly compressible pure carbon dioxide and the stationary phase was hydrophobic, bonded phase silica of questionable purity. Since 1995, the perception of SFC has changed drastically with the popularity of pcSFC now at an all time high; while open tubular column SFC has been only able to meet the original expectations [1].

Today pcSFC is known to be a separation technique that is similar to HPLC. Similar hardware and software are employed as HPLC. SFC almost always uses carbon dioxide as the main component in the mobile phase in conjunction with both a co-solvent modifier and a chemical additive. Separations are usually performed via a gradient elution schedule where the composition of the mobile phase versus time is monitored. Polar stationary phases such as bare silica as well as silica bonded with cyanopropyl, aminopropyl, diol, and 2-ethylpyridine, functionalities are normally utilized [2]. On the other hand, nonpolar stationary phases currently have minimal utility in pcSFC.

pcSFC has advantages over GC in that nonvolatile, thermally degradable compounds are easily separated. When compared with HPLC, pcSFC is often the faster chromatographic approach for nonvolatile analytes due to the low viscosity of the mobile phase allowing for higher flow rates. Higher speed translates into greater throughput and more samples per day. More rapid re-equilibration due to the lower viscosity and higher diffusivity of the mobile phase means shorter cycle time compared to reversed phase HPLC. Nowadays pcSFC is considered to be predominantly normal phase HPLC

* Corresponding author. Tel.: +1 540 231 6680; fax: +1 540 231 3255.
E-mail address: ltaylor@vt.edu (L.T. Taylor).

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with a compressible fluid and thus is orthogonal to reversed phase HPLC. Column pressure drop is low; while peak resolution is high with pcSFC. Solvent consumption and waste generation are low since the carbon dioxide is easily removed at atmospheric pressure [3]. The most popular detectors are ultraviolet diode arrays with high pressure flow cells, evaporative light scattering devices, and mass spectrometers. For chiral analytical method development, pcSFC is the ideal technique for small drug-like racemic molecules versus the much more tedious chiral HPLC approach. The technology is also readily scaleable and very popular in the pharmaceutical industry. Numerous chiral stationary phases that have historically been developed for HPLC application can be easily screened in the SFC mode.

Achiral pcSFC is not nearly as advanced as chiral pcSFC because analytes are diverse and often more polar coupled with the fact that there are few suitable polar stationary phases available. The situation however is changing. For example, a new highly successful analytical SFC/MS strategy has recently been designed, developed, and implemented in an industrial setting. Success in the selection of five stationary phase chemistries (i.e. diol, 2-ethylpyridine, diethylaminopropyl, dinitrophenyl, and benzenesulfonamide) for screening was reflected in more than 85% of target compounds being resolved from their impurities in a research mixture [4].

The future of pcSFC will no doubt ultimately rest upon its applicability to achiral separations of highly polar analytes. Until recently, this goal was thought to be unrealistic because carbon dioxide-based fluids were deemed to be incompatible with water soluble, ionic analytes or high molecular weight compounds. In other words, such concepts as ion exchange SFC, ion pair SFC, hydrophilic interaction SFC, or a combination thereof were uncommon [5]. A complementary but less severe situation concerning separation of highly polar analytes developed in reversed phase HPLC where it was shown that conventional stationary phases failed to create sufficient retention for isolation of hydrophilic and uncharged compounds [6,7].

Hydrophilic interaction liquid chromatography (HILIC) was developed to deal with retention of very polar analytes. The existence of a water-enriched layer on the polar stationary phase under aqueous conditions, and a partitioning of analytes between a mobile phase and the "wet" stationary phase continue to be a basic and accepted mechanism for HILIC in the liquid chromatography community. HILIC mode separations were first published in 1975 where carbohydrates were separated by an amino-silica phase with a mobile phase of acetonitrile and water (75:25). Since the HILIC acronym was first suggested by Alpert in 1990, the number of publications has increased most notably since 2003 [8]. Therefore, for the liquid chromatographer, the separation of polar hydrophilic compounds is best achieved with polar stationary phases and partly aqueous mobile phases with the separation mode being termed HILIC. Nevertheless, HILIC is a type of liquid chromatography and all the disadvantages of HPLC follow such as high viscosity, low diffusivity, large column pressure drop, and thermal column gradients. To enjoy the benefits afforded by pcSFC, one might then question whether HILIC-SFC is feasible?

If one reflects on the situation generally believed to exist today in most pcSFC experiments with modified carbon dioxide based fluids, HILIC-like conditions would seem to exist although traditionally, current pcSFC technology does not employ aqueous-based mobile phases. For example, a non-aqueous HILIC-like retention situation for pcSFC would be supported by the work of Parcher et al., 20 years ago [9]. In their experiments, monolayers of carbon dioxide and methanol adsorption were measured on both bare silica and octadecylsilica stationary phases in the presence of 100% CO_2 and CO_2 modified with methanol. The amount of CO_2 and MeOH adsorbed varied with CO_2 density and was greatest near the critical point of CO_2. Based upon these observations, Berger later suggested that when mobile phase components adsorb onto stationary phases; they form a dense, liquid-like film on the surface [10]. He speculated that this film is usually denser than the components in the mobile phase, but the film consists of the same chemical species. If the stationary phase is covered with a denser, more polar film of adsorbed mobile phase, Berger reasoned that pcSFC must almost always be a normal phase technique where partitioning between the more dense (i.e. adsorbed) and less dense (i.e. mobile phase) components occurs.

Employment of a ternary mobile phase would introduce yet another adsorbed component (i.e. additive) to the stationary phase. If that third component were water, all the ingredients would be in place for HILIC-like chromatography where the organic phase is CO_2; while water being more polar than the modifier is preferentially adsorbed to either the bare silica or bonded phase sites near the silica. A separation mode can thus be envisioned where a polar stationary phase (i.e. bare silica) attracts and is enriched by the more polar part of the eluent (i.e. water) that acts as the retentive element. The mobile phase at the same time offers reasonable solvent properties to allow a fast and linear distribution between the two phases.

We wish to report a study wherein HILIC-SFC has been employed to separate not only classical model mixtures of polar analytes but also the elution of ten drug-like molecules of interest to the pharmaceutical industry. High purity bare silica coupled with methanol modified CO_2 containing a small percentage of water characterizes the mobile phase. Depending on the nature of the polar analytes, an additional additive was incorporated to suppress ionization. Trifluoroacetic acid (TFA) was used for acids, isopropylamine (IPAm) for bases, and ammonium acetate (AA) as a buffer salt for neutrals. The chromatographic results of the study make clear the critical nature of water in the mobile phase for both polar compound retention and selectivity. Bare silica from two vendors has been employed in all experiments.

2. Experimental

2.1. Materials

ACS grade caffeine, ibuprofen, cortisone, hydrocortisone, sulfanilamide, sulfamethoxazole, sulfaguanidine, sulfamerazine, sulfamethazole, sulfamethoxine, thymine, cytosine, prednisone, uracil, estriol, theophyline, thiamphenicol, indomethacin, warfarin, carbamazepin, acetazolamide, fenofibrate, haloperidol, omeprazole, pimozide, niflumic acid, isopropyl amine (IPAm), trifluoroacetic acid (TFA), and ammonium acetate (NH_4OAc) were obtained from Sigma–Aldrich (St. Louis, MO). Molecular structures for most of these compounds are found in Fig. 1. Methanol and H_2O were HPLC grade and were obtained from Fisher Scientific (Pittsburgh, PA). Bare porous silica columns (150 mm × 4.6 mm, d_p = 5 μm) were provided by both Princeton Chromatography Inc. (PrincetonSFC Lot Number: 20106633S) (Cranbury, NJ) and Waters Corp. (Viridis SFC Part Number: 186004910) (Milford, MA). Bare silica dimensions were stated for Princeton to be 60 Å average pore diameter, 1.15 mL/g pore volume, 519 m^2/g and for Waters to be 93 Å average pore diameter, 0.88 mL/g pore volume, 338 m^2 g surface area.

2.2. Standard mixtures

Three mixtures of polar standards were prepared to evaluate the bare silica columns under pseudo HILIC-SFC conditions. A mixture (labeled B) of six sulfonamides was employed with IPAm for column stability testing; while for TFA a mixture (labeled A) of estriol, cortisone, hydrocortisone, ibuprofen, and prednisone

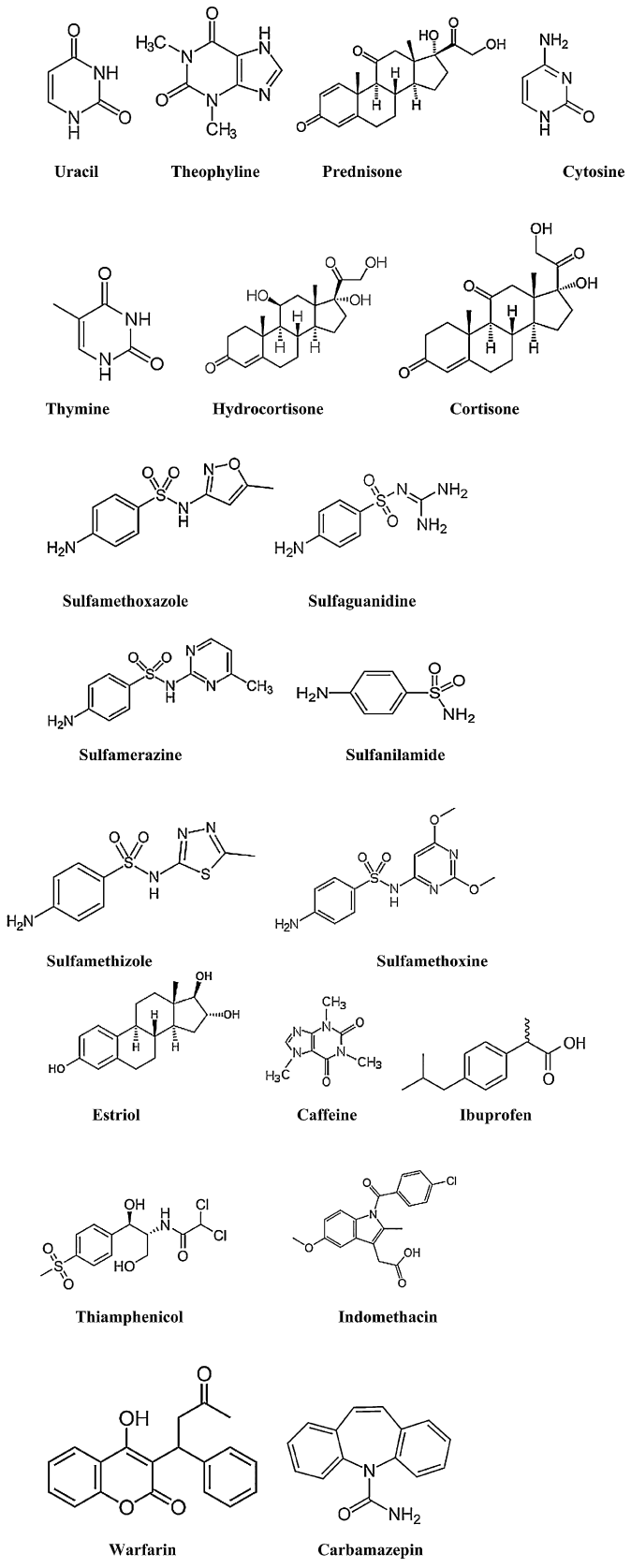

Fig. 1. Chemical structure of standards and drug molecules.

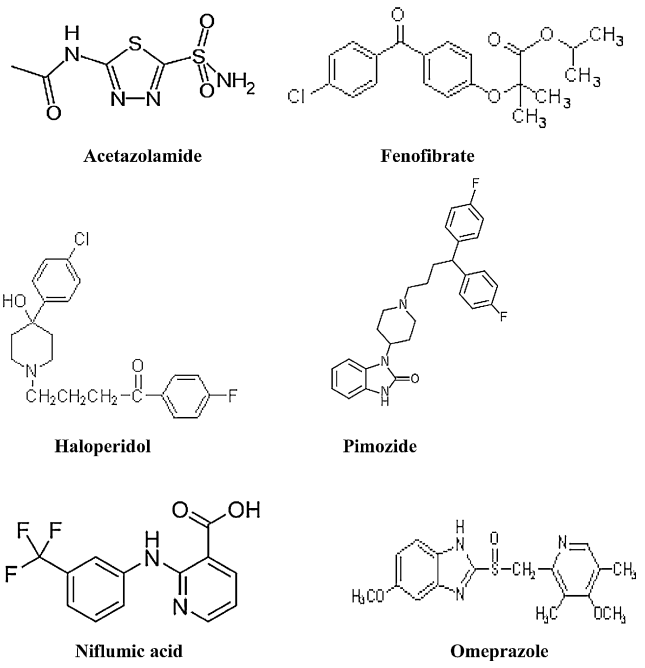

Fig. 1. (*Continued*)

was used. A mixture (labeled C) of caffeine, cortisone, hydrocortisone, thymine, cytosine, prednisone, uracil, and theophyline was used for buffer + water and water alone additives. Solutions of all drug-like molecules (thiamphenicol, indomethacin, warfarin, carbamazepin, acetazolamide, fenofibrate, haloperidol, omeprazole, pimozide, and niflumic acid) were prepared as single compounds. The concentration of each compound regardless of the solution was approximately 1 mg/mL in MeOH.

2.3. SFC/UV analyses

A Waters Corp. (Milford, MA) SFC equipped with high pressure pump, diode array, auto-sampler, oven heater set to 60 °C, and back pressure regulator set at 140 bar was employed. All SFC/UV analyses at 254 nm were performed via gradient elution using the following program:

Time (min)	% A	% B
0	95	5
1	95	5
9	50	50
10	50	50
11	95	5
15	95	5

A = CO_2, B = MeOH + 5% H_2O + additive (10 mM NH_4OAc, 0.2% TFA, or 0.5% IPAm). Liquid flow rate: 4 mL/min.

2.4. Injection protocols

For each stability-timed study, 8 new bare silica columns (4 Waters and 4 Princeton columns dedicated to each of the four additive combinations) were evaluated. The following protocol was followed for each column/additive combination regardless of the column source and with water in the mobile phase. First, blank solvent (i.e. MeOH) was injected into the bare silica column followed by duplicate injections of a MeOH solution of the specific mixture earmarked for the pre-selected additive. Between injections, the column was re-equilibrated four minutes (i.e. 11–15 min as stated above in the mobile phase schedule) with 95% A/5% B. Later in the study, the re-equilibration time was reduced to as low as one minute without an elution problem. At the end of the analysis

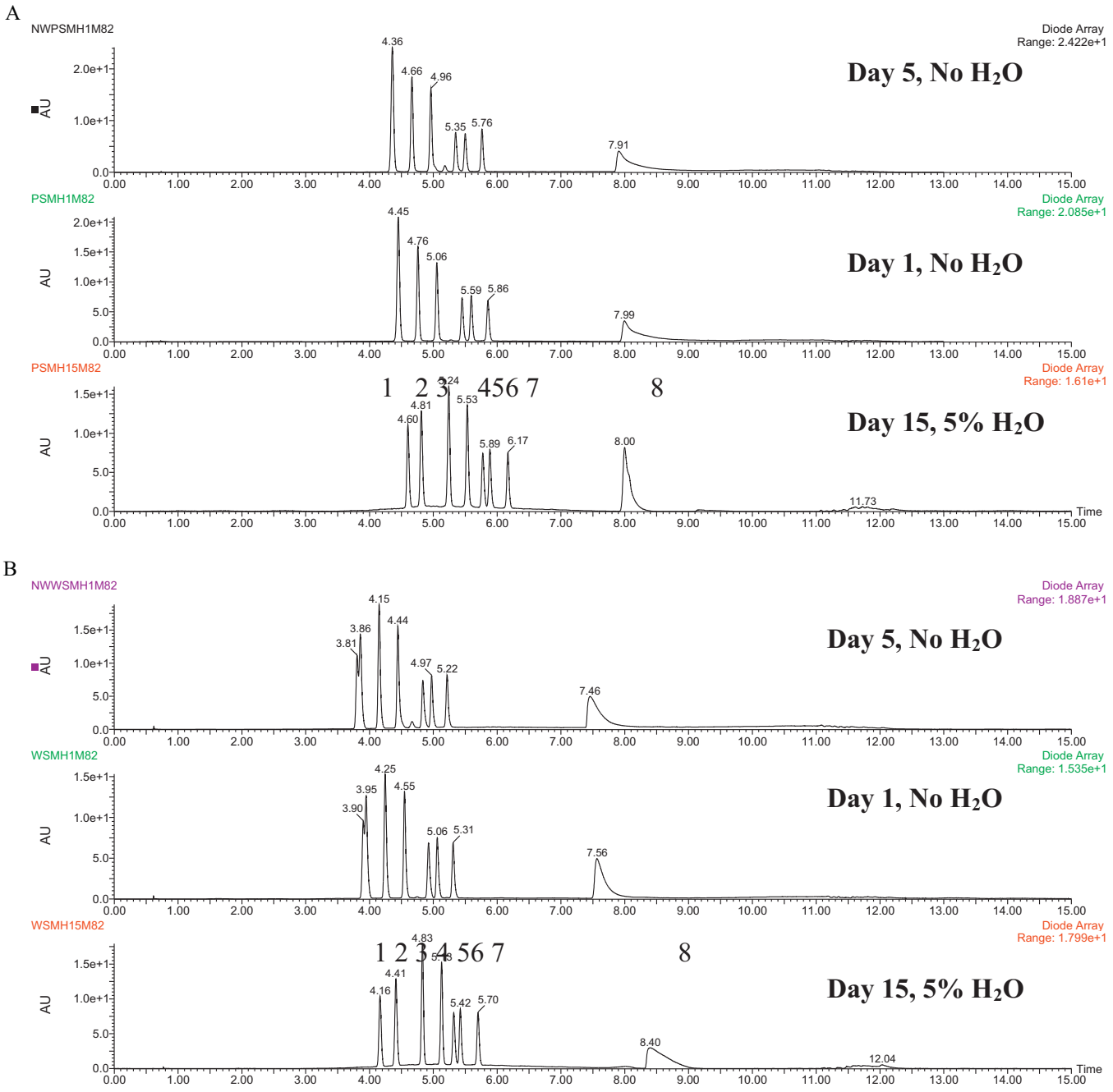

Fig. 2. A Selected chromatograms for (A) Princeton Chromatography and (B) Waters Corp. bare silica separation of mixture N. The traces compare the separation with water in the mobile phase (lowest SFC) with water absence from the mobile phase (top two SFC's).

day when the column was to be stored, a standard wash procedure was followed. Specifically, the column was flushed with 50/50 MeOH/CO_2 for 10 min at 4 mL/min follow by 100% CO_2 for 5 min. After the last injection for the day and the corresponding wash procedure, the column was capped until the next stability-timed point. When changing mobile phase additives, blank MeOH was first employed followed by duplicate injections of the new mixture assigned to the new additive. Between injections, the column was re-equilibrated as before.

Chromatographic traces over a 5-day period of the same three mixtures coupled with the four additives but without water in the mobile phase were generated approximately 30 days after the initial 15 day study. The same protocol was used except water was not present in the mobile phase. After the Day 1 injection, the column was washed and stored as usual. Then a Day 3 injection was made, and this time the column was not washed as before. The experiment was then terminated with a Day 5 injection.

3. Results and discussion

3.1. Overview

The use of water as a mobile phase additive in pcSFC with a bare silica stationary phase and a gradient of CO_2/MeOH mobile phase have been studied. Water, and in some cases, a secondary additive was mixed with the MeOH prior to gradient delivery. The primary focus was to (a) observe changes in selectivity when water is added to the mobile phase, (b) assess reproducibility of retention when

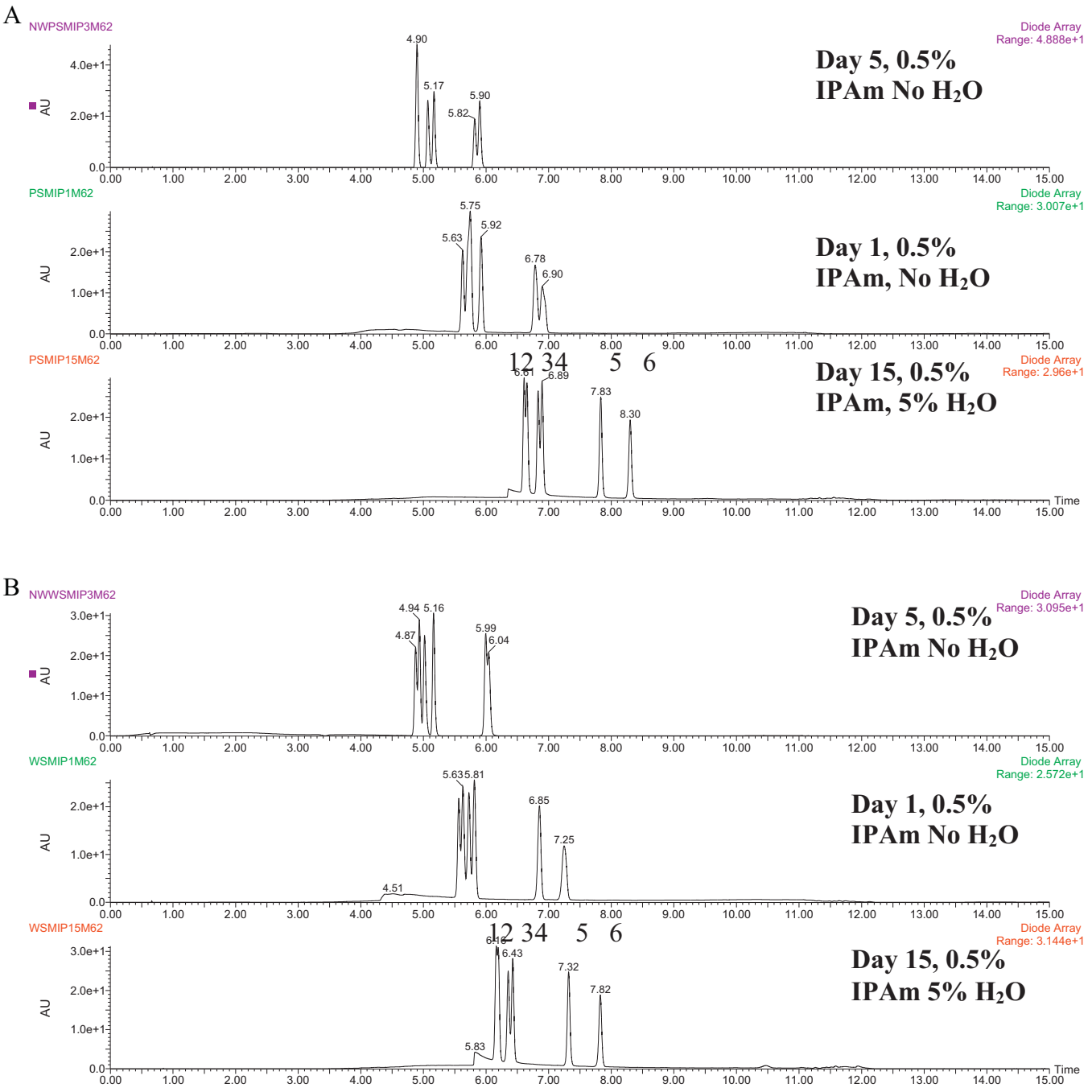

Fig. 3. A Selected chromatograms for (A) Princeton Chromatography and (B) Waters Corp. bare silica separation of mixture B. The traces compare the separation with water in the mobile phase (lowest SFC) with water absence from the mobile phase (top two SFC's).

water is included in SFC mobile phases, and (c) observe if the use of water as an additive permanently alters the stationary phase. Virgin packed bare silica columns from two vendors were employed for separation of three test mixtures and ten drug-like compounds with four additive combinations. All columns were evaluated on Days 1, 3, 5, 10, 15 during the 15-day schedule using the same SFC instrument, temperature, mobile phase composition, and mobile phase gradient schedule. Each column was re-equilibrated and stored between runs following the same identical procedure which is outlined in the Experimental section. At the outset, it was clear with each of the four additives that 5% water does not render the bare silica columns useless. Rather, water enhances the separation of each of the three polarity mixtures. A HILIC-like retention mechanism is envisioned wherein the silica is solvated by the more polar component (i.e. water) in the mobile phase. Analyte partitioning is hypothesized to take place between the more dense adsorbed water component and the less dense water component of the mobile phase. The reproducibility of each separation over the 15-day period regardless of the additive when water is introduced into the modifier is striking. The rapid column re-equilibration with bare silica from both vendors after gradient elution was far superior to what is thought to be observed with conventional normal phase liquid chromatography. Exemplary chromatograms that illustrate the study are shown in Figs. 2–5. Retention times (RT), peak width at half height (PWHH), and peak asymmetry (PA) for duplicate injections on Days 1 and 15 are listed in Tables 1 and 2. A brief discussion of the results obtained via each chromatographic protocol follows.

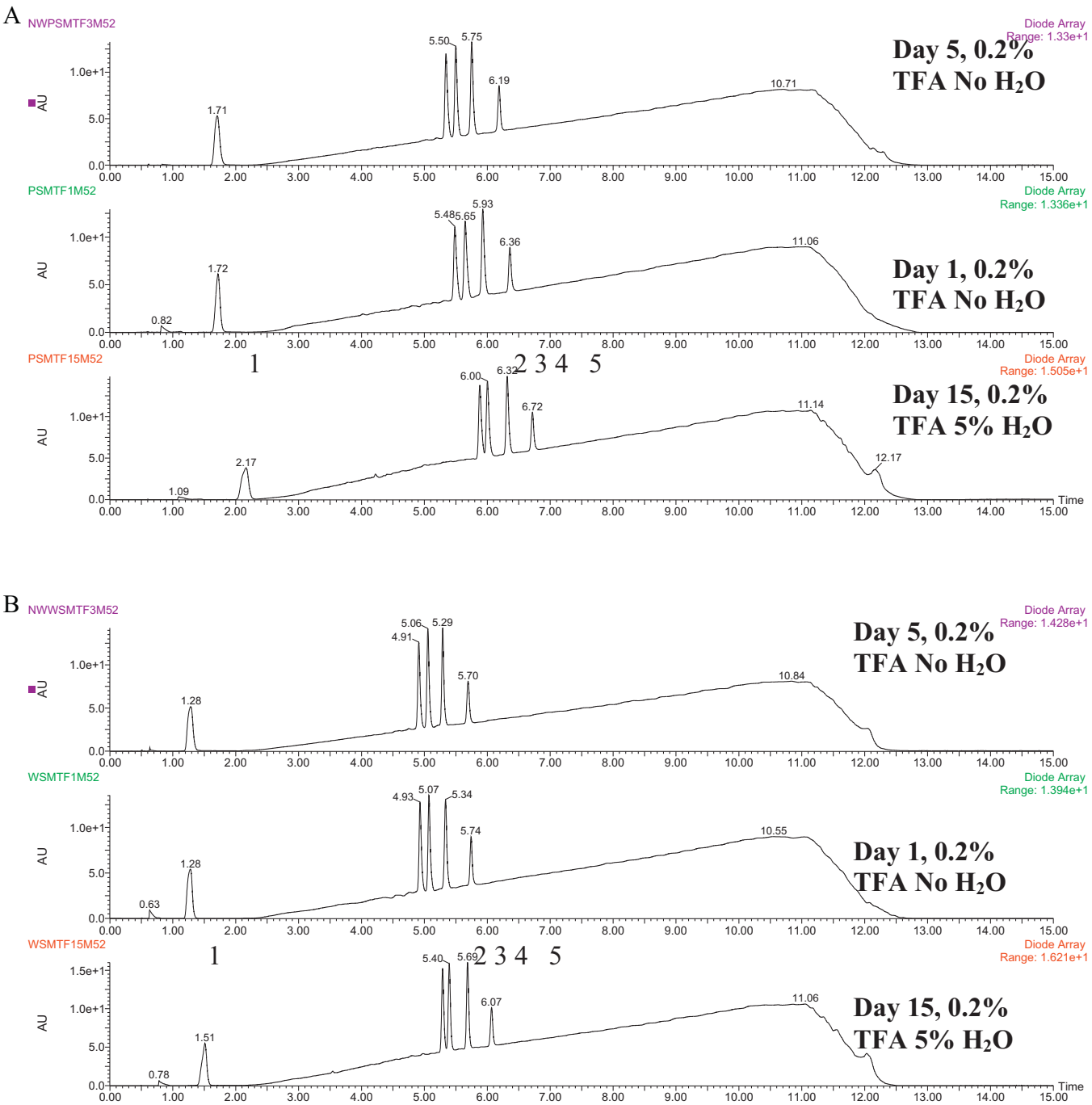

Fig. 4. A Selected chromatograms for (A) Princeton Chromatography and (B) Waters Corp. bare silica separation of mixture A. The traces compare the separation with water in the mobile phase (lowest SFC) with water absence from the mobile phase (top two SFC's).

3.2. Additive = 5% H_2O

Initially, the stability of silica columns from Princeton Chromatography and Waters Corp. was investigated using 5% H_2O in methanol-modified CO_2 over a 15-day period. Table 1 (left side) lists the average retention times for each component in the three mixtures for the two vendor columns. Fig. 2A and B shows the SFC/UV trace for separation of a mixture of 8 "neutral" compounds on the 15th day of evaluation using bare silica from Princeton Chromatography and Waters Corp. Baseline resolution was achieved for all eight compounds with water as the only additive. The retention time, peak width at half height, and peak asymmetry of the first 7 eluting compounds did not change significantly from Days 1 to 15 for both columns. See Table 2. PWHH and PA, although, were slightly greater in most cases for the Princeton column versus the Waters column. An insufficient number of injections, however, were made to determine the significance of this difference. Only the cytosine peak shape changed from Day 1 (when it was sharp) to Day 15 where it became much broader with each succeeding day. This behavior for cytosine was observed with both vendor columns using 5% H_2O in MeOH; whereas the other seven compounds consistently behaved in a straightforward manner as their polarity, solubility, and basicity were all similar. The elution behavior of the poorly water-soluble cytosine was unsatisfactory and more variable than the other analytes; and thus may have been been more of a factor than its unique primary amine functionality

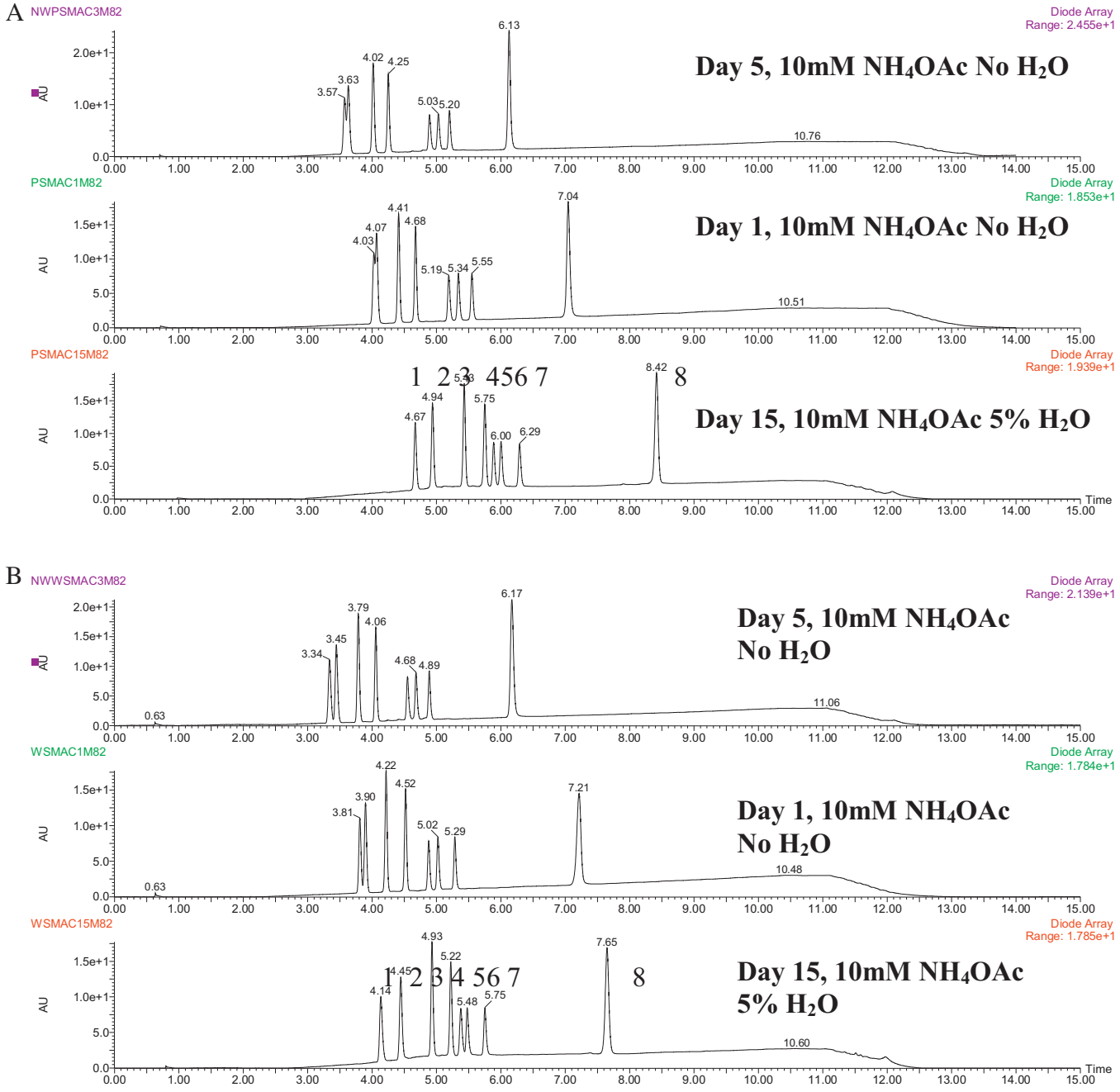

Fig. 5. A Selected chromatograms for (A) Princeton Chromatography and (B) Waters Corp. bare silica separation of mixture N. The traces compare the separation with water in the mobile phase (lowest SFC) with water absence from the mobile phase (top two SFC's).

in comparison with the other analytes in the mixture. Elution order and peak assignments were as follows: 1-caffeine, 2-theophyline, 3-thymine, 4-uracil, 5-cortisone, 6-prednisone, 7-hydrocortisone, and 8-cytosine.

The study next addressed the issue of bare silica chromatographic integrity in the absence of water in the mobile phase. The columns used were the same ones as used previously for water in the mobile phase. Duplicate injections were made after washing with 50/50 MeOH/CO_2 for 10 min and 100% CO_2 for 5 min followed by storing the column for an indefinite period. Each mixture composition and analyte concentration were the same as the study containing water. The gradient elution schedule was also identical to the original study. The stability of silica columns from Princeton Chromatography and Waters Corp. was investigated using only methanol-modified CO_2 over a 5-day period. The chromatographic behavior was very similar from Days 1 to 5. Fig. 2A and B shows the SFC/UV trace for separation of the mixture of 8 compounds on Days 1 and 5 of the evaluation. Comparison of the chromatograms, however, in either Fig. 2A or B revealed significant changes in the earliest two eluting peaks when water was present and absent in the mobile phase. First, a sharper response for cytosine when water was removed from the mobile phase was observed. Next, weakly basic caffeine and theophyline were baseline resolved with water (R_S = 1.67 (Pr) and 2.91 (Wa)); while the two compounds on both bare silica columns co-eluted in the absence of water on Days 1–3–5.

Table 1
Retention times for the separation on Days 1 and 15 of three polarity mixtures (i.e. neutral (N), acidic (A), basic (B)) with four modifier/additive combinations on bare silica from two vendors with water in and absent from the mobile phase. Pr = Princeton Chromatography, Inc., Wa = Waters Corp., RT = retention time.

Mixture N (H_2O only)	RT (min) Days 1 and 15 (Princeton)	RT (min) Days 1 and 15 (Waters Corp.)	Mixture N (No additive)	RT (min) Days 1 and 15 (Princeton)	RT (min) Days 1 and 15 (Waters Corp.)
Caffeine	4.63 and 4.60	4.22 and 4.16	Caffeine	4.45 and 4.36	3.90 and 3.81
Theophyline	4.84 and 4.81	4.46 and 4.41	Theophyline	4.45 and 4.36	3.95 and 3.86
Thymine	5.28 and 5.26	4.87 and 4.83	Thymine	4.76 and 4.66	4.25 and 4.15
Uracil	5.60 and 5.53	5.17 and 5.13	Uracil	5.06 and 4.96	4.55 and 4.44
Cortisone	5.76 and 5.78	5.35 and 5.32	Cortisone	5.44 and 5.36	
Prednisone	5.88 and 5.89	5.45 and 5.42	Prednisone	5.59 and 5.49	5.06 and 4.97
Hydrocortisone	6.18 and 6.17	5.72 and 5.70	Hydrocortisone	5.86 and 5.76	5.31 and 5.22
Cytosine	8.30 and 8.00	7.92 and 8.40	Cytosine	7.99 and 7.91	7.56 and 7.46
Mixture N ($NH_4OAc + H_2O$)	RT (min) Days 1 and 15 (Princeton)	RT (min) Days 1 and 15 (Waters Corp.)	Mixture N (NH_4OAc only)	RT (min) Days 1 and 15 (Princeton)	RT (min) Days 1 and 15 (Waters Corp.)
Caffeine	4.63 and 4.67	4.12 and 4.14	Caffeine	4.03 and 3.59	3.81 and 3.34
Theophyline	4.91 and 4.94	4.43 and 4.45	Theophyline	4.07 and 3.63	3.90 and 3.45
Thymine	5.39 and 5.43	4.90 and 4.93	Thymine	4.41 and 4.02	4.22 and 3.79
Uracil	5.72 and 5.75	5.21 and 5.22	Uracil	4.68 and 4.25	4.52 and 4.06
Cortisone	5.83 and 5.89	5.33 and 5.38	Cortisone	5.19 and 4.90	4.95 and 4.61
Prednisone	5.95 and 6.00	5.43 and 5.48	Prednisone	5.34 and 5.03	5.02 and 4.68
Hydrocortisone	6.24 and 6.29	5.72 and 5.75	Hydrocortisone	5.55 and 5.20	5.29 and 4.89
Cytosine	8.44 and 8.42	7.73 and 7.65	Cytosine	7.04 and 6.13	7.21 and 6.17
Mixture B ($IPAm + H_2O$)	RT (min) Days 1 and 15 (Princeton)	RT (min) Days 1 and 15 (Waters Corp.)	Mixture B (IPAm only)	RT (min) Days 1 and 15 (Princeton)	RT (min) Days 1 and 15 (Waters Corp.)
Sulfamethazole	6.64 and 6.61	6.21 and 6.16	Sulfamethazole	Co-eluted	5.56 and 4.87
Sulfamethoxine	6.69 and 6.65	6.24 and 6.19	Sulfamethoxine	Co-eluted	5.64 and 4.94
Sulfamerazine	6.86 and 6.83	6.40 and 6.35	Sulfamerazine	Co-eluted	Co-eluted
Sulfanilamide	6.94 and 6.89	6.49 and 6.43	Sulfanilamide	5.92 and 5.17	6.64 and 5.16
Sulfamethoxazole	7.90 and 7.83	7.41 and 7.32	Sulfamethoxazole	6.76 and 5.82	6.59 and 5.99
Sulfaguanidine	8.44 and 8.30	7.95 and 7.82	Sulfaguanidine	6.90 and 5.90	7.25 and 6.04
Mixture A ($TFA + H_2O$)	RT (min) Days 1 and 15 (Princeton)	RT (min) Days 1 and 15 (Waters Corp.)	Mixture A (TFA only)	RT (min) Days 1 and 15 (Princeton)	RT (min) Days 1 and 15 (Waters Corp.)
Ibuprofen	2.55 and 2.17	1.51 and 1.51	Ibuprofen	1.72 and 1.71	1.28 and 1.26
Cortisone	6.13 and 5.88	5.29 and 5.29	Cortisone	Co-eluted	4.93 and 4.91
Prednisone	6.25 and 6.00	5.40 and 5.40	Prednisone	5.65 and 5.50	5.07 and 5.06
Hydrocortisone	6.69 and 6.32	5.70 and 5.69	Hydrocortisone	5.93 and 5.75	5.34 and 5.29
estriol	7.04 and 6.72	6.07 and 6.07	estriol	6.36 and 6.19	5.74 and 5.70

3.3. Additive = 0.5% IPAm + 5% H_2O

Bare silica columns from both vendors were next evaluated for separation of basic analytes with a mobile phase additive mixture composed of 0.5% IPAm + 5% H_2O in MeOH as the modifier. Fig. 3A and B shows pcSFC/UV separations on Day 15 of mixture B with the two vendor columns. Baseline resolution of compounds 5 and 6 was readily achieved as long as water was in the mobile phase; whereas sulfamethazole and sulfamethoxine were only partially resolved in the mixture. A similar situation was observed with sulfamerazine and sulfanilamide which nearly co-eluted on each of the five-day duplicate injections. As before, the study demonstrated excellent reproducibility day after day in the presence of the binary additives. Retention times with the Waters column were approximately 30 s shorter for each compound than the Princeton column. For each of the analytes that were resolved, PWHH and peak asymmetry approximated the results obtained with mixture N. It is important to remember that after each study, the column was flushed with 50/50 CO_2/MeOH for 10 min with the assumption that water and basic additives would be removed followed by 100% CO_2 for a 5 min re-equilibration. The elution order was 1-sulfamethazole, 2-sulfamethoxine, 3-sulfamerazine, 4-sulfanilamide, 5-sulfamethoxazole, and 6-sulfaguanidine.

Fig. 3A and B also shows the separation of the same sulfonamide mixture during a 5-day period using only 0.5% IPAm as the additive (i.e. no water). Contrary to mixture N, water as the only additive in the modifier yielded much greater selectivity in mixture B for the elution of the last two compounds rather than the initial two compounds. For example, sulfamethoxazole and sulfaguanidine are baseline resolved with water, but they are only barely resolved without water. Bare silica from both vendors without water gave a much poorer separation of these two compounds, but of the two, the Waters Corp. bare silica proved to be better. While retention times were constant with water over the 15 days, retention times of each component without water decreased with each day's injection over the 5-day period.

3.4. Additive = 0.2% TFA + 5% H_2O

Virgin silica columns from both vendors were evaluated with an additive mixture of TFA and water for elution of mixture A which contained five acidic compounds. Fig. 4A and B shows results for each silica column on the 15th day of evaluation. Baseline resolution of the five analytes was observed with ibuprofen eluting very early with poor peak shape as evidenced by the large PWHH with both vendor columns. Again, both columns showed good reproducibility in the presence of TFA and H_2O. Peak asymmetries were comparable to the analytes in mixtures N and B. The elution order was: 1-ibuprofen, 2-cortisone, 3-prednisone, 4-hydrocortisone, and 5-estriol.

Fig. 4A and B also shows the separation of mixture A with only 0.2% TFA as the additive. For these chromatographic parameters, the data with and without water appeared to be very similar unlike the situation with mixtures N and B. Component 1 (ibuprofen)

Table 2
See caption for Table 1. PWHH = peak width half height; PA = peak asymmetry.

	PWHH (s) Day 1 (Pr)	PWHH (s) Day 15 (Pr)	PWHH (s) Day 1 (Wa)	PWHH (s) Day 15 (Wa)	PA Day 1 (Pr)	PA Day 15 (pr)	PA Day 1 (Wa)	PA Day 15 (Wa)
Mixture N (H_2O only)								
Caffeine	2.46	2.46	2.64	2.64	1.33	1.33	1.33	1.33
Theophyline	2.82	2.88	2.52	2.52	1.00	1.33	0.75	1.00
Thymine	2.52	2.52	2.34	2.34	1.00	1.33	1.07	1.00
Uracil	2.46	2.58	2.16	2.28	1.33	1.33	1.20	1.00
Cortisone	2.52	2.58	2.40	2.46	1.20	1.00	1.14	1.33
Prednisone	2.76	2.58	2.34	2.40	1.33	1.00	0.88	1.33
Hydrocortisone	2.58	2.46	2.28	2.34	1.33	1.20	1.33	1.33
Cytosine	6.06	6.18	60.60	222.00	1.83	4.00	2.33	7.43
Mixture N ($NH_4OAc + H_2O$)								
Caffeine	2.58	2.46	2.76	3.06	1.25	1.13	1.00	1.25
Theophyline	2.46	2.46	2.64	3.06	0.83	1.25	1.00	1.00
Thymine	2.40	2.40	2.76	2.58	1.00	1.13	1.00	1.00
Uracil	2.34	2.40	2.34	2.46	1.00	1.00	1.00	1.43
Cortisone	2.52	2.46	2.58	2.70	1.50	1.50	1.50	1.25
Prednisone	2.70	2.58	2.88	2.52	1.50	1.50	1.67	1.00
Hydrocortisone	2.82	2.64	2.58	2.58	1.50	1.50	1.00	1.67
Cytosine	2.82	3.66	3.90	4.20	1.00	0.86	1.00	0.87
Mixture B (IPAm + H_2O)								
Sulfamethazole	CE	CE	CE	CE	CE	CE	CE	CE
Sulfamethoxine	CE	CE	CE	CE	CE	CE	CE	CE
Sulfamerazine	3.00	2.88	3.00	2.70	1.33	CE	1.00	CE
Sulfanilamide	3.00	3.00	3.00	2.88	1.14	CE	0.75	CE
Sulfamethoxazole	3.00	3.18	3.00	3.30	1.00	1.00	1.00	1.00
Sulfaguanidine	3.60	3.42	3.60	3.60	1.00	1.00	0.89	1.20
Mixture A (TFA + H_2O)								
Ibuprofen	8.70	8.70	4.20	6.60	0.85	0.58	0.50	0.67
Cortisone	3.48	3.00	2.40	2.46	1.75	1.75	0.89	0.89
Prednisone	3.54	3.12	2.52	2.70	2.00	1.75	0.87	1.00
Hydrocortisone	3.54	3.06	2.52	2.70	2.00	1.50	1.00	1.13
estriol	3.06	3.00	2.70	2.76	1.20	1.33	1.43	1.13

eluted very early with water as a broad peak, but without water, a much sharper peak was observed. Also, the studies without water revealed a much smaller shift to lower retention time for the mixture in going from Days 1 to 5 than for the other two mixtures. Peak shape and resolution seemed to be comparable with and without water in the mobile phase for both vendor columns.

3.5. Additive = 10 mM NH_4OAc + 5% H_2O

Mixture N which contained the same eight compounds mentioned previously was examined a second time with 10 mM ammonium acetate and 5% water as co-additives. NH_4OAc was chosen because compounds that exhibit late elution and strong peak tailing peak shapes when pure MeOH was used showed dramatically improved chromatographic behavior in the presence of this salt additive [11]. Fig. 5A and B shows results of this study measured on Day 15. Again, both columns showed excellent stability in the presence of this additive mixture. The elution order was unchanged from the earlier study with only water as the additive: 1-caffeine, 2-theophyline, 3-thymine, 4-uracil, 5-cortisone, 6-prednisone, 7-hydrocortisone, and 8-cytosine. In this case, the cytosine peak eluted at approximately the same time, but the peak remained sharp throughout the study in contrast to the results that were observed when only water was used as the additive. There were significant vendor differences however. Retention times for the Waters column were shorter, but PWHH's were greater especially for cytosine, Table 2. Peaks were slightly sharper with salt as the co-additive in comparison with data taken when only water was the additive.

As in the previous study with TFA and no water, retention time with salt and no water of each component decreased nearly half a minute in going from Days 1 to 5 for both the Princeton Chromatography and Waters Corp. columns, Fig. 5A and B. Resolution of caffeine and theophyline appears to be impaired somewhat with the Princeton Chromatography column; whereas resolution is more apparent with the Waters column with no water in the mobile phase. There is little doubt that water enhances resolution, however, regardless of the vendor as nearly baseline separation of each component is observed.

3.6. Re-introduction of water to the mobile phase

It was of interest to know if the incorporation of water into the mobile phase would restore the excellent selectivity and peak shapes that were initially observed. Fig. 6 describes this study with mixture N. The top trace (with water, Day 15) is the reference chromatogram for the neutral mixture with water and ammonium acetate as the additives. The bottom trace (now with water, inj. #1) is the results of the first injection after the study with no water in the mobile phase. Peaks are well resolved, but peak retention times are the same as with the "no water" results. The intermediate traces which continue the experiment are repeats of the same injection with water in the mobile phase. Peak shapes are unchanged but retention time drifts to longer times consistent with the initial result (top trace) obtained with the bare silica. One concludes that while removal of water alters selectivity for specific analytes and retention time for all analytes in mixture N, it does not destroy the chromatographic integrity of bare silica. Furthermore, re-institution of water into the mobile phase restores the excellent chromatographic behavior of bare silica for the separation of highly polar analytes.

3.7. Elution behavior of individual drug molecules

The elution behavior of ten different drug molecules (Fig. 1) using the three different additives mixtures and water alone from

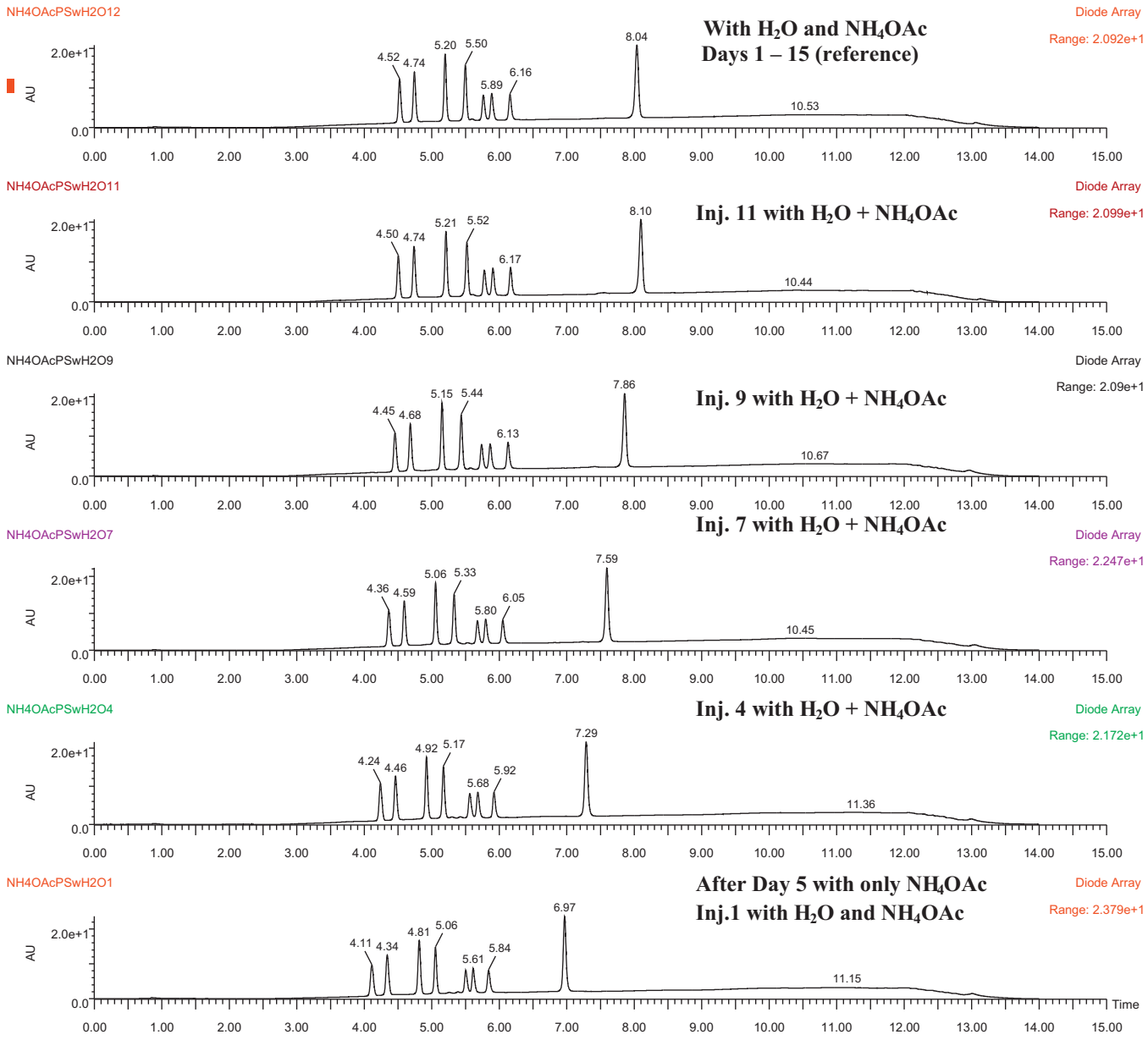

Fig. 6. Elution of mixture N. Effect of adding 5% water and 10 mM ammonium acetate back to the mobile phase after a 5-day period where no water was incorporated into the mobile phase.

the same virgin, bare silica columns was investigated. Additives were identical to those used in the initial study. Table 3 lists the retention time, PWHH, and PA for each of the compounds. Comparable SFC/UV traces with bare silica from the two different vendors for all ten analytes using the various additives were observed. All ten molecules even though they possessed quite different chemistries eluted as sharp peaks from the bare silica columns when either 0.5% IPAm + 5% water or 10 mM NH_4OAc + 5% water were used as additive mixtures. Inspection of Table 3 however will reveal that retention times for the ten compounds differed considerably with these two additive combinations. Either of these two separation conditions may indeed qualify as generic mobile phases for achiral chromatography. On the other hand, haloperidol, pimozide, and omeprazole eluted with tailing and distorted peak shape when 0.2% TFA + 5% water and just 5% H_2O were used with MeOH; while the other seven molecules even yielded sharp peaks with these two additives. In other words, 100% of the randomly selected drugs yielded sharp peaks and short retention times with two of the aqueous mobile phases (IPAm and AA); while 70% of the drug molecules produced sharp peaks and short retention times with aqueous TFA and water alone.

Finally, it is important to note that the re-equilibration time between injections can easily be reduced from 4 min which was universally used in these studies to 1 min as evidenced by the traces shown in Fig. 7.

This work lends credence to an earlier reported study from our laboratory [12] concerning the elution of water soluble nucleobases from silica bonded diol, cyanopropyl, and 2-ethylpyridine stationary phases (instead of bare silica) facilitated with alcohol-modified CO_2 and water as the polar additive. In this work, the common elution pattern exhibited by each of the three stationary phases suggested that water had altered the surface chemistry of the packed stationary phase thus fostering a HILIC-like retention mechanism. In each case, peaks were sharper with water in the mobile phase.

Table 3
Chromatographic parameters for elution of individual drug molecules with bare silica from two vendors employing four difference modifier/additive combinations. For abbreviations see captions for Tables 1 and 2.

Compound	Additive	RT (min)	PWHH (s)	PA
Acetazolamide (Wa)	0.2% TFA + 5% H_2O	5.68	2.58	1.2
	0.5% IPAm + 5% H_2O	6.27	2.4	1.0
	5% H_2O	5.63	2.4	1.0
	10 mM NH_4OAc + 5% H_2O	5.8	2.46	1.4
Acetazolamide (Pr)	0.2% TFA + 5% H_2O	6.16	2.64	1.8
	0.5% IPAm + 5% H_2O	6.75	2.52	1.2
	5% H_2O	6.06	2.4	1.2
	10 mM NH_4OAc + 5% H_2O	6.25	2.7	1.0
Carbamazepine (Wa)	0.2% TFA + 5% H_2O	4.64	2.94	0.3
	0.5% IPAm + 5% H_2O	4.71	2.88	1.2
	5% H_2O	4.62	2.82	1.0
	10 mM NH_4OAc + 5% H_2O	4.77	3	1.2
Carbamazepine (Pr)	0.2% TFA + 5% H_2O	5.18	3	1.4
	0.5% IPAm + 5% H_2O	5.15	2.7	1.4
	5% H_2O	5.15	2.4	1.0
	10 mM NH_4OAc + 5% H_2O	5.21	2.7	1.0
Fenofibrate (Wa)	0.2% TFA + 5% H_2O	1.26	4.62	1.1
	0.5% IPAm + 5% H_2O	1.1	3.9	3.2
	5% H_2O	1.18	7.8	0.6
	10 mM NH_4OAc + 5% H_2O	1.36	9.6	0.8
Fenofibrate (Pr)	0.2% TFA + 5% H_2O	1.51	9	0.5
	0.5% IPAm + 5% H_2O	1.36	7.8	2.5
	5% H_2O	1.5	8.4	0.9
	10 mM NH_4OAc + 5% H_2O	1.7	10.2	0.7
Haloperidol (Wa)	0.2% TFA + 5% H_2O	8.21	22.2	3.7
	0.5% IPAm + 5% H_2O	5.8	1.14	0.7
	5% H_2O	9.91	45	6.5
	10 mM NH_4OAc + 5% H_2O	6.43	3.12	1.3
Haloperidol (Pr)	0.2% TFA + 5% H_2O	7.94	14.4	8.5
	0.5% IPAm + 5% H_2O	5.39	1.08	1.3
	5% H_2O	8.97	24	1.8
	10 mM NH_4OAc + 5% H_2O	7.26	3	1.0
Indomethacin (Wa)	0.2% TFA + 5% H_2O	3.88	4.32	1.5
	0.5% IPAm + 5% H_2O	6.42	3.66	1.4
	5% H_2O	3.95	4.68	1.3
	10 mM NH_4OAc + 5% H_2O	5.21	3	2.2
Indomethacin (Pr)	0.2% TFA + 5% H_2O	4.43	4.2	1.3
	0.5% IPAm + 5% H_2O	6.92	3.84	1.6
	5% H_2O	4.41	3	1.4
	10 mM NH_4OAc + 5% H_2O	5.74	31.8	1.5
Niflumic acid (Wa)	0.2% TFA + 5% H_2O	3.17	6.6	1.1
	0.5% IPAm + 5% H_2O	5.99	4.2	1.6
	5% H_2O	2.87	7.8	1.8
	10 mM NH_4OAc + 5% H_2O	5.63	4.8	3.1
Niflumic acid (Pr)	0.2% TFA + 5% H_2O	3.58	4.2	1.5
	0.5% IPAm + 5% H_2O	6.43	5.4	2.5
	5% H_2O	3.5	3.6	2.0
	10 mM NH_4OAc + 5% H_2O	6.46	5.4	1.6
Omeprazole (Wa)	0.2% TFA + 5% H_2O	7.84	#VALUE!	#DIV/0!
	0.5% IPAm + 5% H_2O	5.16	3	1.4
	5% H_2O	5.12	3.06	2.2
	10 mM NH_4OAc + 5% H_2O	5.23	3	1.4
Omeprazole (Pr)	0.2% TFA + 5% H_2O	8.43	0	#DIV/0!
	0.5% IPAm + 5% H_2O	5.64	2.94	1.6
	5% H_2O	5.67	2.76	1.6
	10 mM NH_4OAc + 5% H_2O	5.69	30.6	1.2
Pimozide (Wa)	0.2% TFA + 5% H_2O	8.34	17.4	3.5
	0.5% IPAm + 5% H_2O	5.52	2.46	1.0
	5% H_2O	8.02	24.6	6.5
	10 mM NH_4OAc + 5% H_2O	7.77	2.7	1.2
Pimozide (Pr)	0.2% TFA + 5% H_2O	8.28	6	4.4
	0.5% IPAm + 5% H_2O	6.09	2.94	1.3
	5% H_2O	8.32	27	4.6
	10 mM NH_4OAc + 5% H_2O	6.44	3	1.0
Thiamphenicol (Wa)	0.2% TFA + 5% H_2O	5.91	2.7	1.5
	0.5% IPAm + 5% H_2O	6.35	2.88	1.4

Table 3 (*Continued*)

Compound	Additive	Ret. time (min)	PWHH (min)	Peak asymmetry
	5% H_2O	5.85	2.4	1.0
	10 mM NH_4OAc + 5% H_2O	5.96	2.88	1.8
Thiamphenicol (Pr)	0.2% TFA + 5% H_2O	6.42	2.58	1.8
	0.5% IPAm + 5% H_2O	6.8	2.64	1.8
	5% H_2O	6.35	2.52	1.2
	10 mM NH_4OAc + 5% H_2O	6.52	2.94	1.4
Warfarin (Wa)	0.2% TFA + 5% H_2O	4.47	3.6	1.6
	0.5% IPAm + 5% H_2O	6.37	3	1.6
	5% H_2O	4.29	3	1.2
	10 mM NH_4OAc + 5% H_2O	4.92	3.6	1.0
Warfarin (Pr)	0.2% TFA + 5% H_2O	4.82	2.82	1.0
	0.5% IPAm + 5% H_2O	6.91	3	1.7
	5% H_2O	4.7	2.46	1.0
	10 mM NH_4OAc + 5% H_2O	5.34	3	1.2

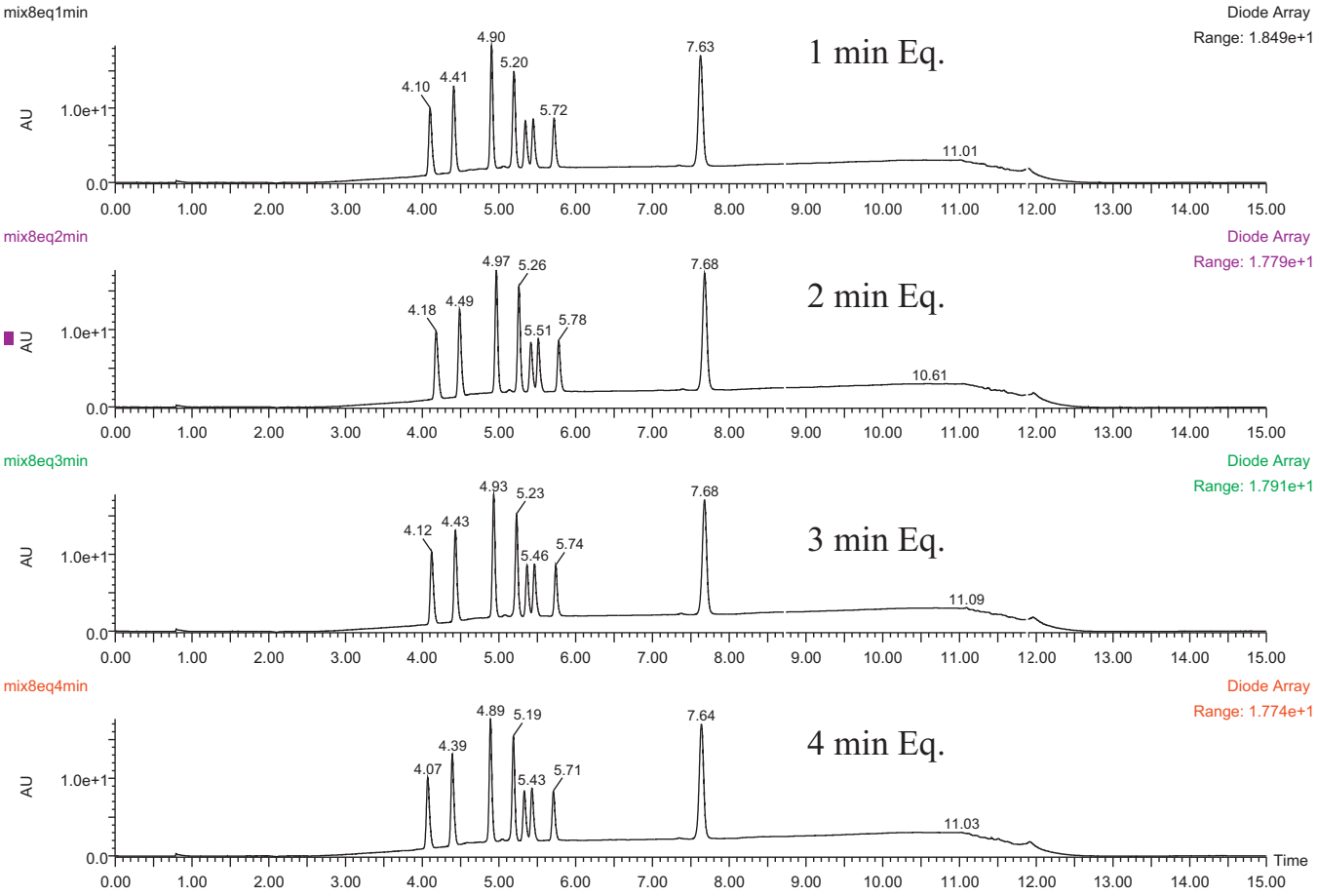

Fig. 7. Effect of equilibrium time between injections on next analysis. Mixture (N), Additives: water + ammonium acetate.

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