Characterization of Enhanced-Fluidity Liquid Hydrophilic Interaction Chromatography for the Separation of Nucleosides and Nucleotides

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Abstract

Hydrophilic Interaction Chromatography (HILIC) is a liquid chromatographic separation mechanism commonly used for polar biological molecules. While nucleotides are very polar analytes, only a few studies have been conducted on their separation using HILIC. Herein, the use of enhanced-fluidity liquid chromatography (EFLC) for the separation of nucleosides and nucleotides under HILIC conditions is investigated. Enhanced-fluidity liquid chromatography involves using common mobile phases with the addition of substantial proportions of a dissolved gas which provides enhanced mobile phase diffusivity and lower viscosity. The impact of varying mobile phase composition: buffer composition, type of base, salt used, salt concentration and mole fraction of CO_2 was studied to provide optimized HILIC separations. Each of these parameters plays a key role in the retention of the analytes, which demonstrates the complexity of the retention mechanism in HILIC. The tailing of phosphorylated compounds was overcome with the use of phosphate buffer and the addition of a strong base; efficiency and peak asymmetry were compared with the addition of either triethylamine (TEA), 1,4-diazabicyclo [2.2.2] octane (DABCO) or 1,5- diazabicyclo [4.3.0] non-5-ene (DBN). DBN and DABCO both led to increased efficiency and lower peak asymmetry; DBN provided the best results. Sodium chloride and carbon dioxide were added to enhance the selectivity between the analytes, hence

nucleosides along with monophosphate nucleotides were successfully separated isocratically with an optimized mobile phase in less than 20 minutes.

I. Introduction

Hydrophilic Interaction Chromatography (HILIC) is a term first introduced by Alpert [1] to describe a variant of normal phase liquid chromatography in which polar stationary phases are used in combination with aqueous/organic mobile phases to separate compounds based on differences in hydrophilicity. In his original paper, Alpert proposed that, when a mobile phase containing a high percentage of organic solvent with a small portion of water is exposed to a hydrophilic stationary phase, a water-enriched layer forms at the surface of the stationary phase. Facile, highly selective separations of peptides, nucleic acids, carbohydrates and other polar compounds are well documented to date using this technique.[1,2]

The exact mechanism involved in the separation has been deeply discussed over the last twenty years. Indeed, Hemstrom *et al.* showed that a surface adsorption model or the combination of surface adsorption and partitioning mechanisms may be more appropriate than the partitioning model to describe the retention of some compounds.[2] However, Alpert's originally proposed model of partitioning between the bulk mobile phase and an adsorbed water-enriched solvent layer on the surface of the stationary phase continues to have strong support. The exact composition of the mobile phase can be tailored to modify all forces involved and enhance the separation.

Acetonitrile is the most commonly used weak solvent in HILIC; nonetheless, replacing it with alcohols has been considered as well.[3, 4, 5, 6, 7, 8, 9] The chromatographic selectivity for compounds using alcohol-based mobile phases differs significantly from that observed with acetonitrile. Also, due to the enhanced polarity of alcohols compared to acetonitrile, the overall

retention decreases as well.[6, 7] Nevertheless, the use of methanol, ethanol or isopropyl alcohol offers incontestable advantages such as enhanced solubility of many analytes and buffers as well as a lowered cost and production of less hazardous waste.[3,4, 10]

Enhanced-fluidity liquid chromatography (EFLC) is a method in which a significant proportion of a miscible gas is dissolved in the mobile phase. This provides a mobile phase that typically retains a solvent strength of similar magnitude to that of the original mobile phase (without the added gas) while significantly increasing the diffusivity and lowering the viscosity of the combined mobile phase. Carbon dioxide is the most commonly used dissolved gas. Previous EFLC studies have documented lower analysis time, increased efficiency and resolution in reversed-phase, normal phase, size exclusion chromatography as well as for chiral separations and critical chromatographic separations of polymers as a direct result of the increased diffusivity and a lower viscosity of enhanced-fluidity mobile phases. [11, 12 , 13, 14, 15] The introduction of carbon dioxide as a weak eluent for HILIC separation of nucleobases was recently investigated by Sandra and coworkers[16] using ethanol/ CO_2 /H_2O mobile phases that were buffered with ammonium formate. Our group recently illustrated the separation of RNA nucleosides by HILIC using a mobile phase of methanol/water/CO_2 mixtures that included an acetate buffer.[17] In both cases, improved chromatographic selectivity was observed with addition of CO_2 to the mobile phase. Alcohol/CO_2/H_2O mixtures were used in both cases and in this study instead of acetonitrile/H_2O/CO_2 mixtures because CO_2 has greater miscibility with alcohol / H_2O mixtures than acetonitrile/ H_2O mixtures. [18] According to the phase diagram of the ternary mixture, at 90/10 (v: v) methanol/water, up to 0.40 mole fraction of CO_2 can be added to the system maintained at or above 60 bar.[18]

The uniqueness of the separation in Hydrophilic Interaction – EFLC motivated us to pursue the subject and begin to deepen our understanding of the mechanism involved in such separations. This was investigated through the separation of nucleotides along with the nucleosides.

Nucleosides and nucleotides are compounds of interest for pharmaceutical chemistry. They have been previously separated under reversed-phase conditions using a porous graphitic carbon stationary phase[19] or by capillary electrochromatography. [20,21] Only a few HILIC studies on the separation of nucleotides[22,23,24] have been published to date and none of these publications have separated the nucleotides along with their nucleosides.

Herein, the separation of adenosine, cytidine, guanosine, uridine and their four monophosphate nucleotides using Hydrophilic Interaction- EFLC is described. The retentivity of diphosphate nucleotides was also studied for the studied conditions to verify the retention trends among the nucleotides.

Many parameters such as buffer composition, buffer concentration, salt type, salt concentration and mole fraction of added CO_2 were varied and optimized to obtain a fast, efficient and selective separation of these compounds. According to the previous Hydrophilic Interaction-EFLC study from our research group, increases in retention and selectivity with the addition of CO_2 are expected. The effects of the different parameters under both LC conditions and with a small proportion of added CO_2 was studied to provide a comparison of their impact in LC and EFLC and obtain the best parameters for an EFLC separation. Results were compared to previously published HILIC separations of nucleotides.

II. Experimental

HPLC Setup

The HPLC system was similar to the previously described setup and assembled from commercially available components.[17] An ISCO 260 D syringe LC pump (Teledyne Isco, Inc. Lincoln, Nebraska) was connected by 0.02 inch stainless steel tubing (VICI Valco Instruments, Houston, TX) to a Valco 6-port 5000 psi injector equipped with a 20 µL injection loop (VICI Valco Instruments, Houston, TX). The injector was connected to the HILIC column: a 4.6 x 150mm XBridge Amide column packed with 3.5 µm particles (Waters, Milford, MA). The column was connected to a Jasco UV-2075 UV-Vis detector with a high-pressure 4-µL flowcell (Jasco Inc. Easton, MD). The wavelength of the detector was set at 262 nm. The outlet of the flow cell was equipped with a 30 µm internal diameter fused silica capillary (Polymicro Technologies, Inc. Phoenix, AZ, USA) of an adjusted length to control the flow and maintain the system pressure.

Chemicals

ACS grade (> 99.8%) anhydrous methyl alcohol was purchased from Mallinckrodt (Phillipsburg, NJ). Ammonium phosphate monobasic and glacial acetic acid were also purchased from Mallinckrodt. Ammonium acetate and sodium acetate were from Jenneile Enterprise (Cincinnati, OH). Sodium chloride, certified ACS, was purchased from Fisher (Thermo Fisher Scientific Inc, Suwanee, GA). Triethylamine was purchased from EM Science (Gibbstown, NJ). Ammonium chloride (99.5%), 1,4-diazabicyclo [2.2.2] octane (98%) and 1,5-diazabicyclo [4.3.0] non-5-ene (98%) as well as all nucleosides and nucleotides were purchased

from Sigma-Aldrich: adenosine (>99%), cytidine (99%), guanosine (>98%), uridine (>99%), adenosine 5'-monophosphate monohydrate from yeast 99% (AMP), adenosine 5'-diphosphate sodium salt from bacteria source 97% (ADP), cytidine 5'-monophosphate disodium salt from yeast 100% (CMP), cytidine 5'-diphosphate sodium salt hydrate from yeast 96.9% (CDP), guanosine 5'-monophosphate disodium salt hydrate from yeast (GMP), guanosine 5'-diphosphate sodium salt 96% (GDP), uridine 5'-monophosphate disodium salt from yeast (UMP), uridine 5'-disodium salt hydrate (UDP). 5.0 Supercritical fluid extraction grade carbon dioxide (99.998 % purity) was purchased from Praxair, Inc (Danbury, CT, USA).

Mobile Phase Preparation

A 90/10 (v/v) methanol/aqueous solution was used for all LC experiments and to prepare the enhanced-fluidity liquid mobile phases. A higher proportion of methanol would compromise the solubility of the analytes and a lower one would limit the amount of CO_2 that can be mixed in the liquid. Therefore, the methanol/water ratio will be held at 90/10 and the composition of the aqueous portion of the mobile phase will be varied to optimize the separation: the choice of buffer, base and other salt added will be discussed in the following sections.

CO_2 was added to the LC mobile phase in a similar fashion as previously described[25] yielding final mole fractions of 0.1, 0.15, 0.18, 0.20 or 0.25 pressurized at 80 bar.[26, 27] All separations were maintained at constant mobile phase flow rate of 0.6 mL/min.

Data Analysis

The retention factor, k, efficiency, N, and asymmetry factor, A_s,[28] for each chromatographic band were determined using exponentially modified Gaussian peak fitting

algorithm using Peakfit version 4 (SPSS Inc. Chicago, IL, USA). Toluene was used as the neutral marker.

III. Results and Discussion

Choice of buffer

Buffers or ionic salts are commonly added to the mobile phase in HILIC to adjust the pH and the ionic strength of the mobile phase as well as the charge state of the analytes.1 Acetate buffers are commonly used in HILIC[2, 29, 30]due to their high solubility in organic buffers and the hydrophobicity of acetate that enables significant retention. [31] Our previous study on hydrophilic interaction-EFLC showed satisfactory results using acetate buffers for the separation of RNA nucleosides.[17] For this study, both sodium acetate and ammonium acetate buffers were considered with concentration varying from 20 to 200mM but all led to long retention times with broadened and tailed peaks for the nucleotides. Phosphate buffers were chosen as possible alternatives because previous studies have shown value in their use in the separation of biomolecules by HILIC.[1, 3, 9] The phosphate buffer concentration was varied between 25 and 75mM. As shown in Figure 1, both the efficiency and the retention factor of nucleotides increase with increasing phosphate buffer concentration. The increases in retention factor and efficiency are larger between 25 mM and 50 mM than between 50 mM and 75mM. The slower increase in efficiency between 50 and 75 mM seems to indicate that the gain in efficiency that could be expected from working at higher concentrations would not be significant. Because of the limited solubility of phosphate buffer in methanol/water, we chose not to work at concentrations higher than 75 mM to prevent any precipitation or clogging of the system. For the remainder of the

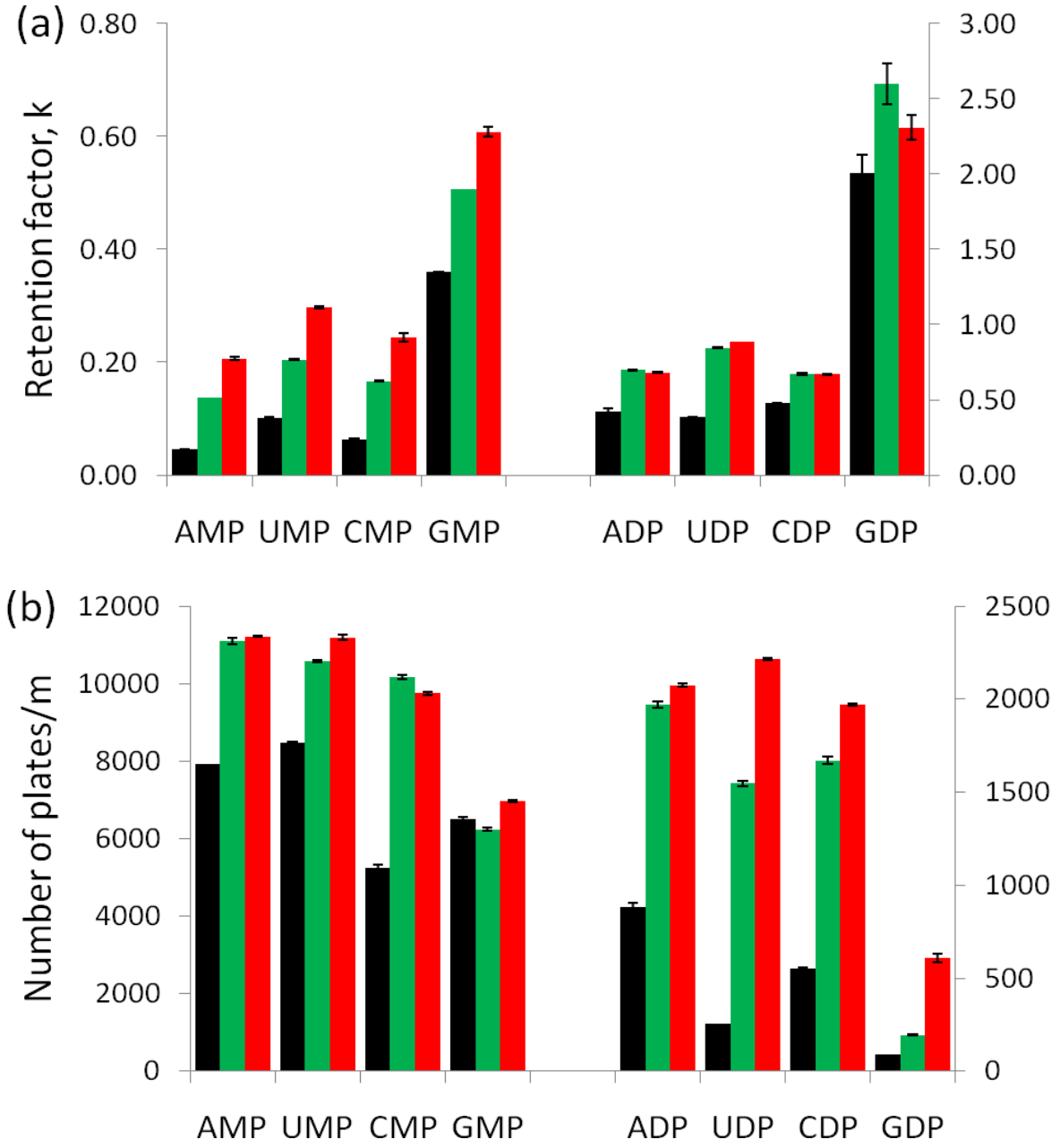

Figure 1: Effect of buffer concentration on the nucleotides: (a) change in retention factors, (b) change in efficiencies. The y-axis on the left corresponds to the values for the monophosphate compounds, the axis on the right for the diphosphate compounds. Conditions: (90:10) ethanol/ammonium phosphate with 25 mM ammonium phosphate (■), 50 mM ammonium phosphate (■), 75 mM ammonium phosphate (■)

study, ammonium phosphate concentration was maintained at 75mM in the aqueous phase, which has a pH of 4.4.

Effect of base addition

Even with the use of ammonium phosphate buffer, the poor efficiency of the nucleotides, which was primarily caused by peak tailing, remained a challenge. Triethylamine (TEA) has been frequently used as a mobile phase modifier to reduce tailing of Lewis basic analytes. [32, 33]

Two bases that are widely used in organic synthesis and described as superbases due to their strong basicity in both aqueous and organic solvents were also considered and compared to TEA: 1,4-diazabicyclo [2.2.2] octane (DABCO) and 1,5- diazabicyclo [4.3.0] non-5-ene (DBN).

A study on the impact of base concentration between 5 and 20mM on the efficiency was performed and did not show a significant impact; therefore all bases were added at the same concentration of 5mM into the aqueous buffer. The impact of base addition was studied both in LC and EFLC for which a 0.1 mole fraction of CO_2 was chosen. Figure 2 compares the efficiency of the nucleotides obtained with TEA, DABCO and DBN in the mobile phase which contained ammonium dihydrogen phosphate buffer. When compared to the data where no base was added, DBN provides the best results with a 12 to 18% increase in efficiency in LC for monophosphate nucleotides and 111% to 320 % for the diphosphates. In EFLC, the efficiency increased from 9 to 39% for monophosphates, and from 67 to 115 % for diphosphates. Tailing was the major contributor causing low efficiency for the phosphorylated compounds; the impact of base addition on the asymmetry of the peak was studied to better understand the impact of each base on efficiency.

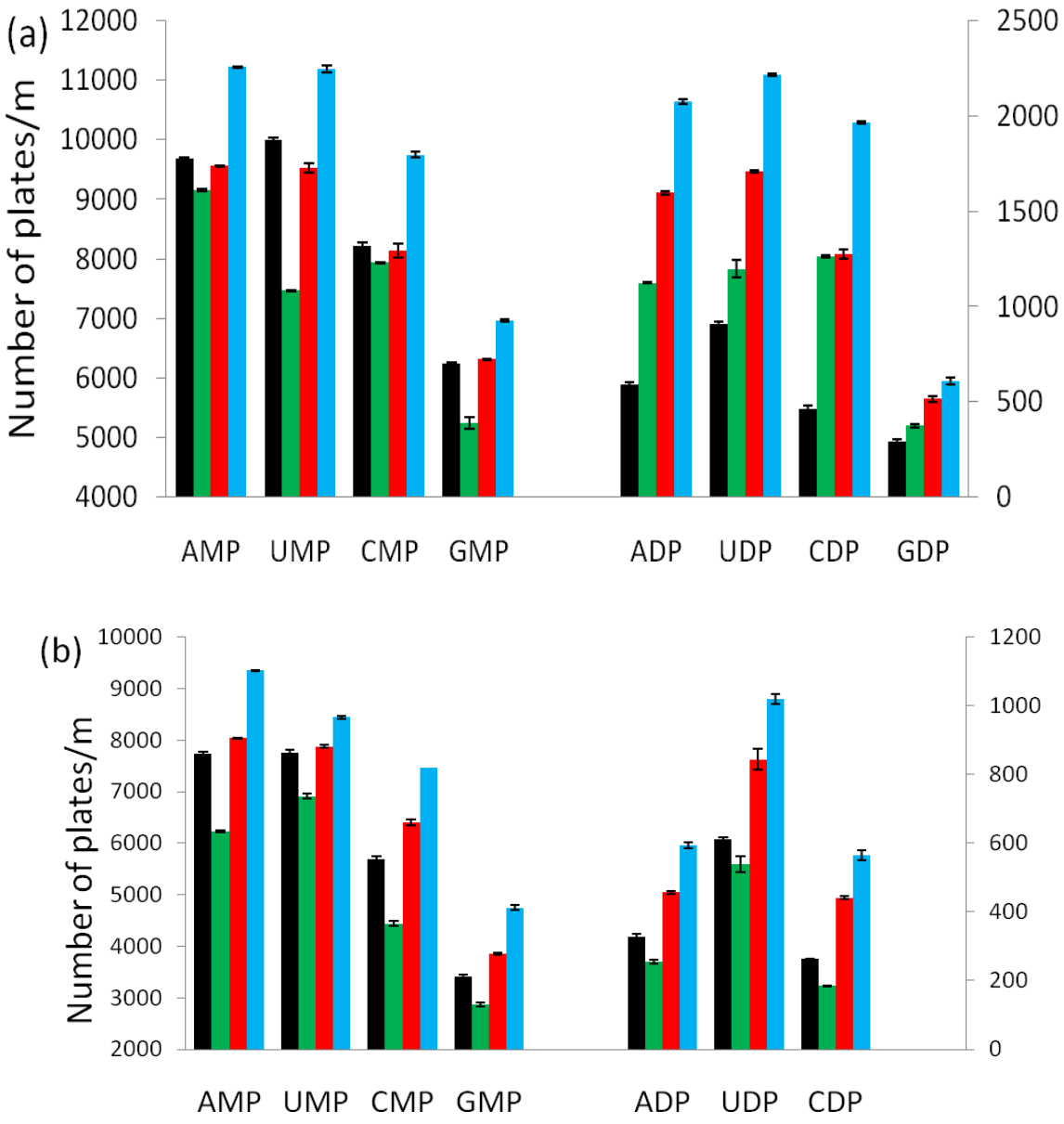

Figure 2: Effect of different bases on the efficiency of nucleotides. Conditions: 5mM of each base was added to a 75 mM ammonium phosphate solution, which was used to prepare the 90/10 (v/v) methanol/aqueous mobile phase (a). in LC and (b). with 0.1 mole fraction CO_2 added. The y-axis on the left corresponds to the efficiency values for the monophosphate compounds, the axis on the right to the values for the diphosphate compounds. No base (■), TEA (■), DABCO (■), DBN (■).

Figure 3 shows that DBN provided both the greatest decrease in peak asymmetry and increase in efficiency, followed by DABCO. Both bases greatly improved the peak shape while marginally affecting the retention of the analytes; no significant decrease in k was observed in EFLC and an average of 10% decrease in k was observed in LC. On the other hand, the addition of TEA either increased the peak asymmetry or did not impact it significantly as seen in Figure 3; but TEA also led to lowered retention with an average decrease in k of 16% in EFLC and 40% in LC, which decreased the overall efficiency even when the peak asymmetry remained constant.

Table 1 compares, in both water and acetonitrile, the Bronsted[34] and hydrogen bond basicities[35] of ammonium (the cation in the buffer solution), TEA, DABCO and DBN. According to these values, DBN is the strongest base on all of the considered scales; DBN and DABCO are classified as superbases due to their strong Bronsted basicity; and DBN, DABCO, TEA and ammonium are respectively classified as very strong, strong, and medium hydrogen-bond bases.[36] The basicities in Table 1 also clearly highlight concepts that are often neglected in chromatographic system. For example, the Bronsted basicity of compounds can change substantially when moving from one solvent to the next and also the Bronsted basicity is not necessarily correlated with the hydrogen bond basicity.[37,38, 39]

Interestingly, the observed decrease in the asymmetry factor correlates most with the hydrogen-bond basicity of additives. Many have noted the importance of hydrogen-bond interactions in the HILIC separation using amide stationary phases.[1,2,40] Minimizing strong hydrogen bond interactions is clearly important to improving the peak shape of the nucleotides in these separations. Preliminary data on peak shape analysis of nucleotide chromatographic bands using phosphate buffered acetonitrile/H_2O mobile phases also illustrate DBN outperforming TEA in improving peak tailing.[41]

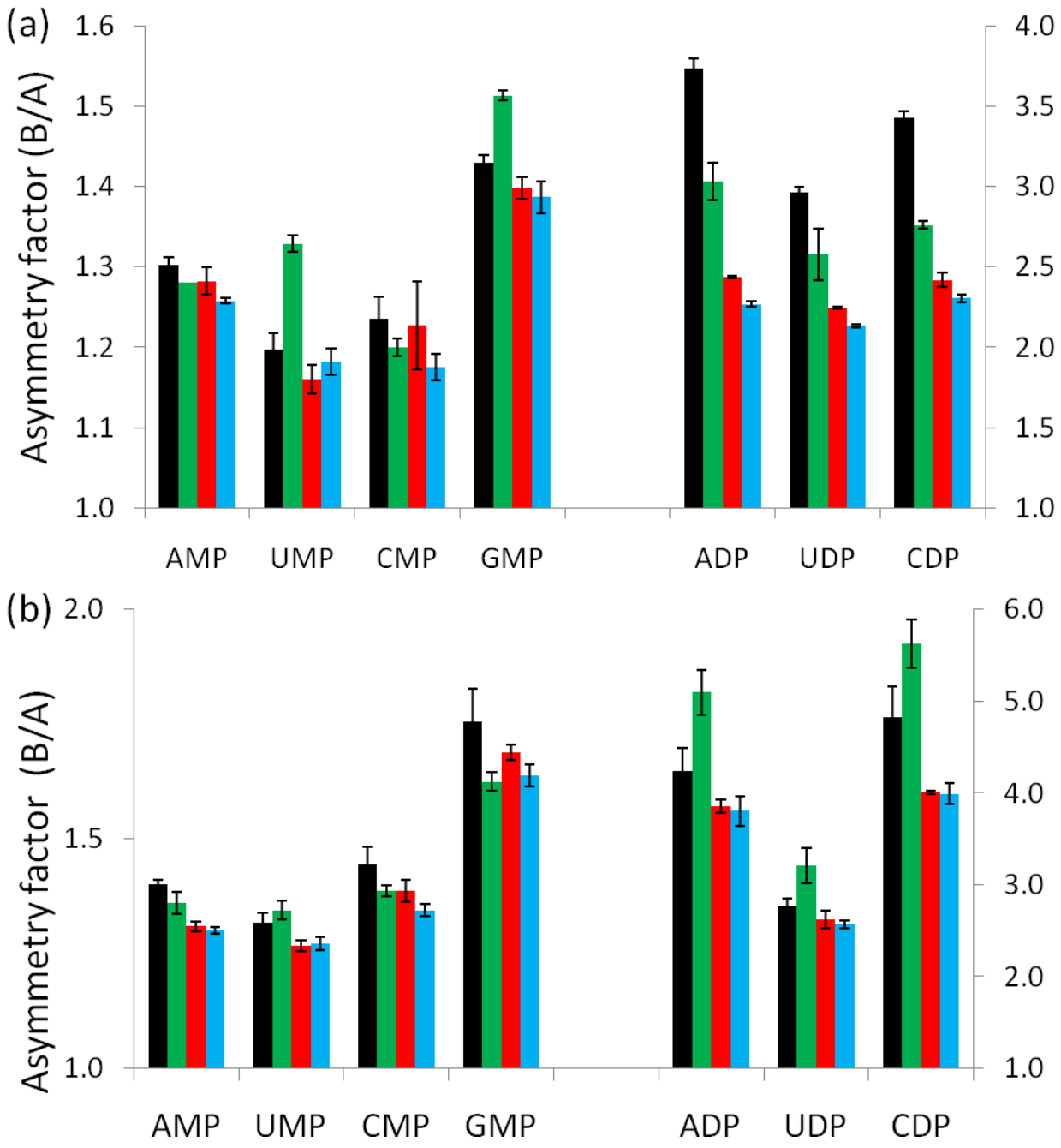

Figure 3: Effect of different bases on the peak asymmetry of nucleotides. Conditions: similar to Figure 2. (a). in LC and (b). with 0.1 mole fraction CO_2 added. The y-axis on the left corresponds to the efficiency values for the monophosphate compounds, the axis on the right to the values for the diphosphate compounds. No base (■), TEA (■), DABCO (■), DBN (■)

	pK_a in water[3]	**pK_a in Acetonitrile**[34]	**pK_{BHX}**[36]	**Hydrogen-bond basicity Classification**[36]
DBN	13.40	23.80	3.8	Very Strong
DABCO	8.80	18.29	2.33	Strong
TEA	10.90	18.46	1.98	Strong
Ammonium	9.23	16.46	1.74	Medium

Table 1: Bronsted pK_a values for bases of in water and acetonitrile and pK_{BHX} of the considered bases

Based on these results, we concluded that the addition of DBN provided the best results in terms of improved peak shape and efficiency. All remaining studies will be conducted with an addition of 5mM DBN to the aqueous phase. The solution of 75 mM ammonium phosphate + 5mM DBN has a pH of 5.5.

Effect of salt addition and salt type

Salt addition to the mobile phase significantly affects retention in HILIC but the specific effects reported in the literature show discrepancies in trends. While Alpert[1] originally suggested increasing salt concentration as an alternative to decreasing the proportion of organic solvent to reduce the elution time of the compounds, both increases and decreases in retention with increasing salt concentration have been reported. [9, 29, 30, 42] Decreased retention with increasing salt concentration usually occurs with an amino phase or ion-exchange column; columns for which electrostatic interaction plays a great role. Increasing the salt concentration increases the ionic strength which shields electrostatic interactions resulting in shorter retention times as was observed previously using an amide stationary phase.[29] The combination of hydrophilic interaction and electrostatic repulsion was described in detail by Alpert [42] and the acronym ERLIC was chosen to designate such type of interactions.

In this study, the stationary phase amide groups are not charged; therefore electrostatic interactions cannot affect the retention of the analytes. However, as illustrated in Figure 4, the addition of NaCl led to increased retention of the nucleotides. Because the increase of retention affects the nucleotides more than the nucleosides, the addition of NaCl greatly benefits the selectivity and the overall resolution of the compounds. Diphosphate nucleotides were strongly

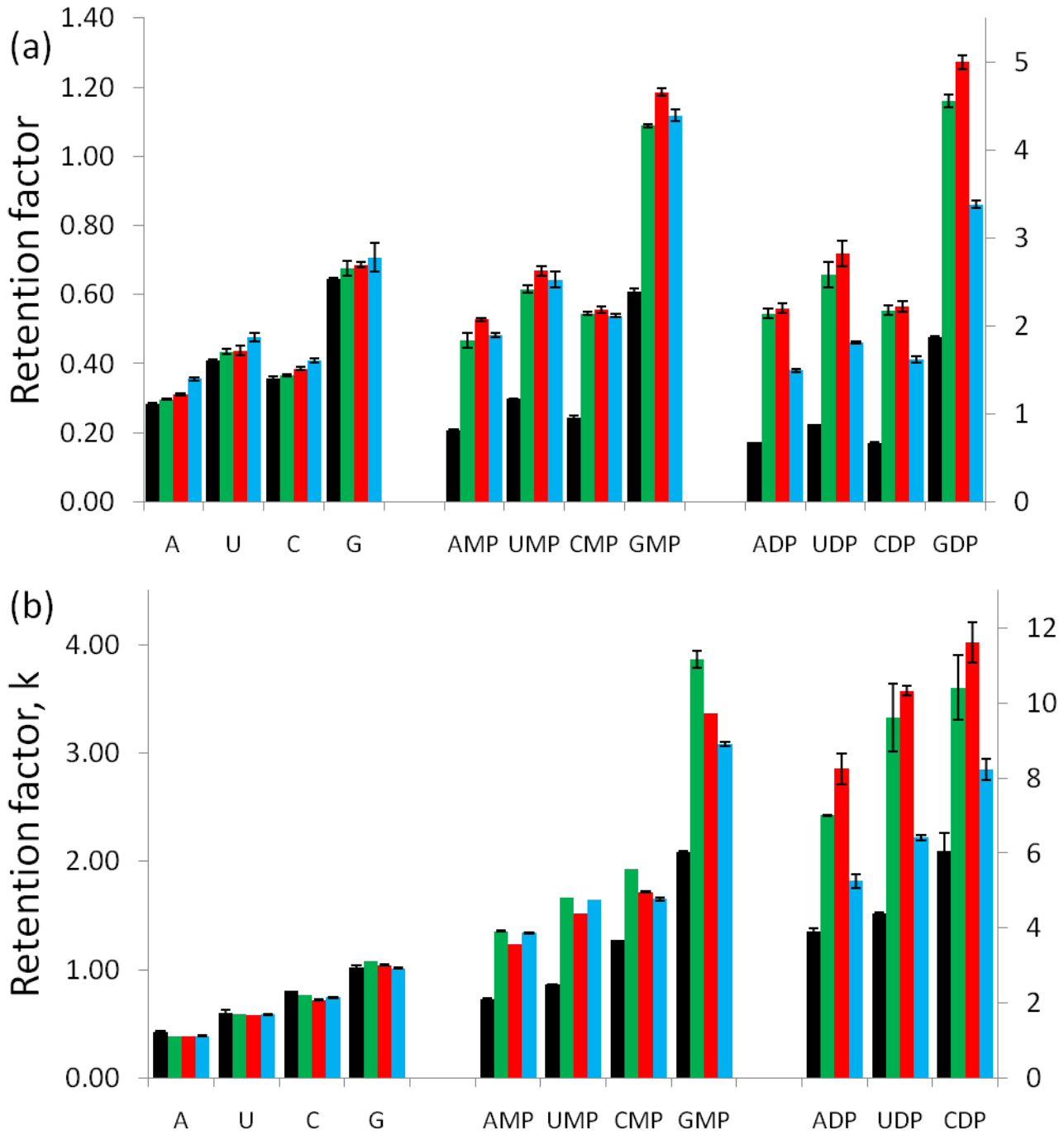

Figure 4: Change in retention factor for different salt added to the mobile phase. (a) in LC, (b) with 0.15 mole fraction CO_2 added. Conditions: (90:10) Methanol/ 75mM ammonium phosphate +5mM DBN +0.2 M salt.The y-axis on the left corresponds to the retention factor values for the nucleosides and monophosphate nucleotides, the axis on the right to the values for the diphosphate nucleotides. No salt (■), NaBr (■), NaCl (■), NH_4Cl (■)

affected by the salt addition; for example, the retention factors in EFLC reached 5 for ADP to 12 for CDP, which is too high for an isocratic separation. A gradient should be considered to separate the diphosphate nucleotides and we will focus on the optimization of the separation of the nucleosides and monophosphate nucleotides for the remainder of this study, which involved only isocratic mobile phases. In this section, the impact of salt concentration and salt type will be studied, while the importance of salt addition for the separation and resolution of all the analytes in EFLC will be detailed in the following section.

When the NaCl concentration is increased from 0 to 0.3 M, a gradual increase in retention of the monophosphates is observed between 33 and 58% at 0.1M, 44 and 77% at 0.2M and 53 to 94% at 0.3M in EFLC as illustrated in S1. The increase in retention with NaCl concentration agreed with the data published previously. Indeed, Nguyen *et al.*[30] and Guo *et al.*[29] both observed increases in retention with increases in salt concentration using uncharged stationary phases. They proposed that salt added to the mobile phase is driven into the immobilized water layer which increases its hydrophilicity and consequently enhances the partitioning and retention of polar analytes.

The impact on retention was compared with addition of other salts with a different cation or anion: NH_4Cl and NaBr at the chosen concentration of 0.2M. While, all three salts led to increased retention of the nucleotides, the specific behavior of the compounds with the different salt added varied slightly. As shown in Figure 4, NaBr has a very similar impact on retention as found with NaCl, while NH_4Cl causes a smaller increase in retention. In LC, the retention of diphosphates increased between 144 and 225% with the addition of NaBr, 167 and 231% with NaCl and between 80 and 142% for NH_4Cl. In EFLC, the increase in retention was not as

remarkable as in LC but still showed an increase between 72 and 118 % with the addition of NaBr, 91 and 135% with NaCl, and 33 and 46% with NH_4Cl for the diphosphates. Both in LC and EFLC, none of the considered salts had a significant impact on the nucleosides. The discrepancy of the impact of salt between nucleosides and nucleotides is responsible for changes in order of elution but more importantly, a great increase in selectivity as seen in S2.

According to the differences in results obtained with the different salt added, it is likely that the hydrophilicity of the liquid layer does not only depend on the salt concentration but also on the size of the ions constituting the layer. Thus, a larger ion would contribute to a greater volume of the water-enriched layer immobilized at the surface of the stationary phase, which would enhance partitioning of hydrophilic compounds, leading to an increase in retention. The results shown above agrees with the theory that ion size influences retention, as the sodium ion is almost twice as large as the ammonium ion, and chloride and bromide ion have a comparable radius. The impact of salt on the retention of the analytes can be used to optimize the separation.

Impact of CO_2 addition

The impact of CO_2 addition to the mobile phase optimized hitherto is studied in more detail. It is of interest to investigate this impact both with and without salt addition. Indeed, both CO_2 and salt increase the retention of the analytes, but their impact on the various analytes differ; therefore a controlled addition of each of these modifiers can lead to enhanced selectivity.

As seen in Figure 5, when no salt is added to the mobile phase, most compounds are co-eluting under LC conditions, and each monophosphate nucleotide elutes faster than its corresponding nucleoside. When CO_2 is added, the retention of the monophosphates is affected more than that of the nucleosides which causes changes in the order of elution for each fraction

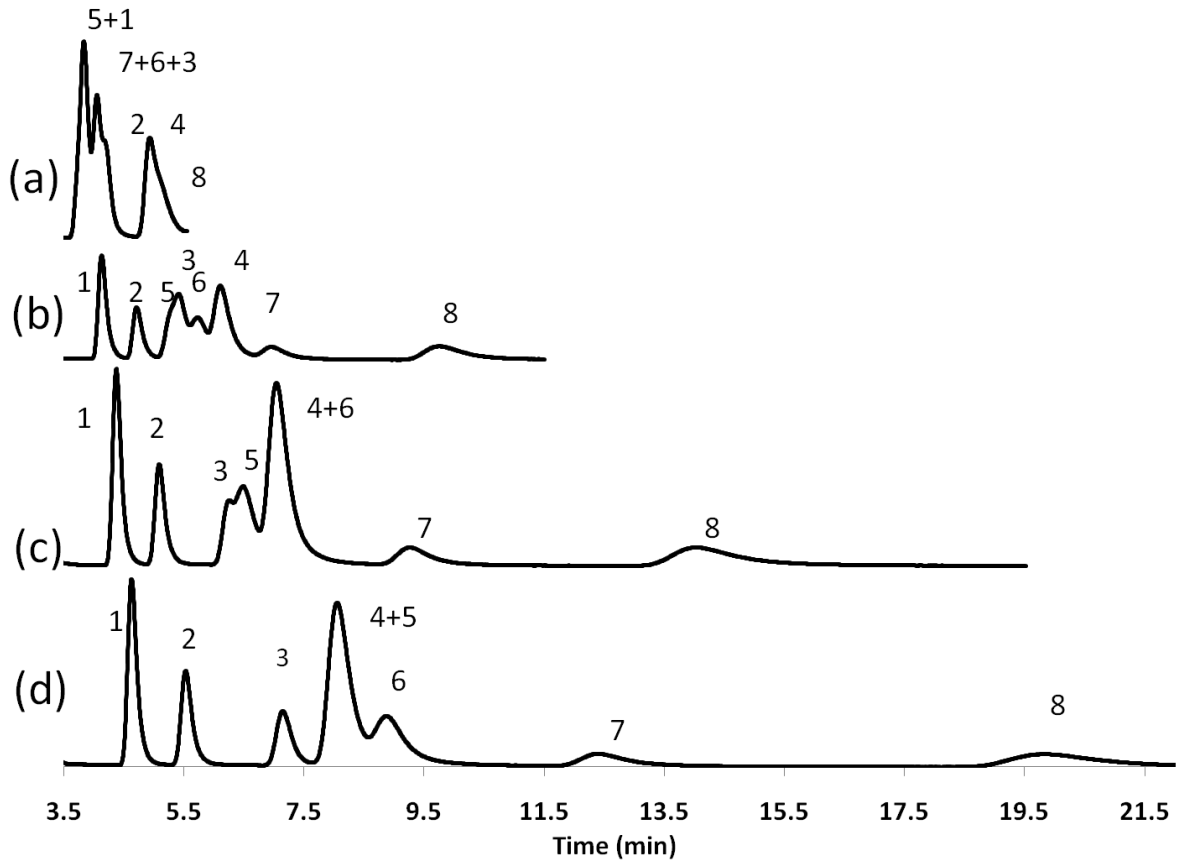

Figure 5: Effect of CO_2 addition. Conditions: (90:10) Methanol/ 75mM ammonium phosphate +5mM DBN. Chromatograms in (a) LC, (b) 0.15 mole fraction of CO_2 (c) 0.20 mole fraction of CO_2, (d) 0.25 mole fraction of CO_2. Adenosine (1), Cytidine (2), Uridine (3), Guanosine (4), AMP (5), CMP (6), UMP (7), GMP (8).

of CO_2 added, as illustrated in Figure 5 b-d. While the addition of CO_2 improved the separation of the eight compounds, selectivity enhancements were still needed.

As discussed in the previous section, the addition of salt has a stronger effect on nucleotides than nucleosides; therefore the monophosphates no longer elute before their nucleosides under LC conditions and the selectivity is much higher as six peaks can be distinguished in Figure 6-a vs three in Figure 5-a. When CO_2 is added to the mobile phase containing NaCl, the selectivity improves greatly as illustrated in Figure 6.

The increase in selectivity results in an increase in resolution. With the addition of 0.18 mole fraction of CO_2, retention factors range between 0.35 and 4.73 (see S3) and the eight compounds are fully separated with resolutions ranging from 1.3 for the UMP/CMP pair to 4.7 for the CMP/GMP pair. The general trend of these results matches our previous results on the study of nucleosides in EFLC[17], but improved mobile phase conditions led to superior results. Indeed, instead of separating four compounds in 12 min, we now separate eight compounds in 16 min at a flow rate of 0.6 mL/min.

As previously discussed[17], efficiency in EFLC with HILIC does not follow the increase observed in many other applications of EFLC [11, 13, 14, 15] which can be explained by the significant increase in retention with CO_2addition. As seen in Figure 7, the efficiency of the monophosphates, which saw a great increase in retention factor, decreases of 35% in average, while the variation in most nucleosides' efficiency is not significant.

As describe above, using methanol as a weak eluent does not give sufficient retention of the compounds to separate the eight compounds of interest. This lack of retention leading to poor resolution has been reported with the use of methanol as a weak eluent.[6, 7] In their review, Hao *et al.* [4] claimed that the strong capacity of methanol to create hydrogen-bonds will compete with

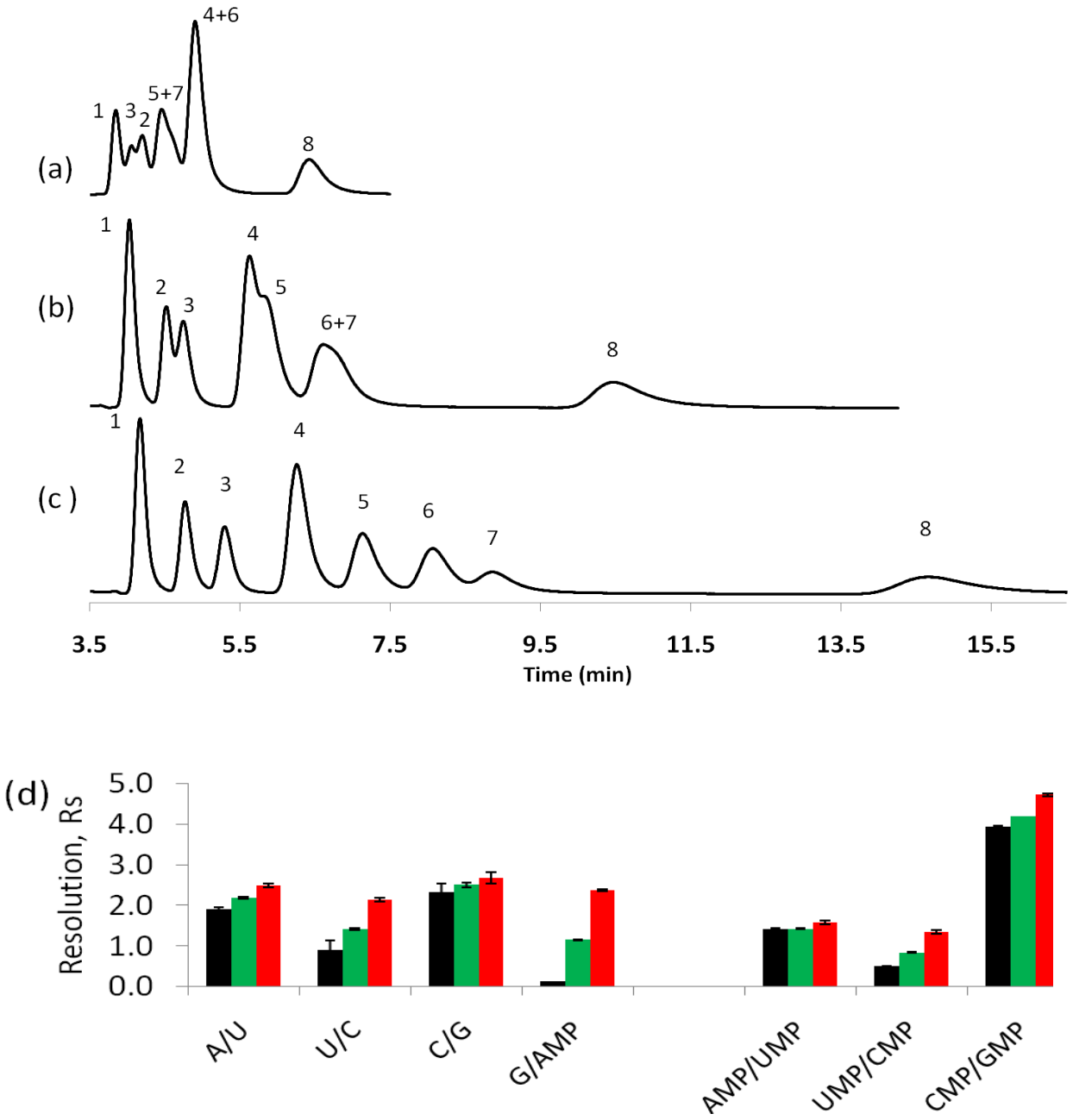

Figure 6: Effect of CO_2 addition in the presence of salt. Conditions: (90:10) Methanol/ 75mM ammonium phosphate +5mM DBN+ 0.2M NaCl. Chromatograms in (a) LC, (b) 0.1 mole fraction of CO_2, (c) 0.15 mole fraction of CO_2, (d). Resolution vs pair of adjacent peaks with 0.1 (■), 0.15 (■), 0.18 (■) mole fraction of CO_2. Adenosine (1), Cytidine (2), Uridine (3), Guanosine (4), AMP (5), CMP (6), UMP (7), GMP (8) .

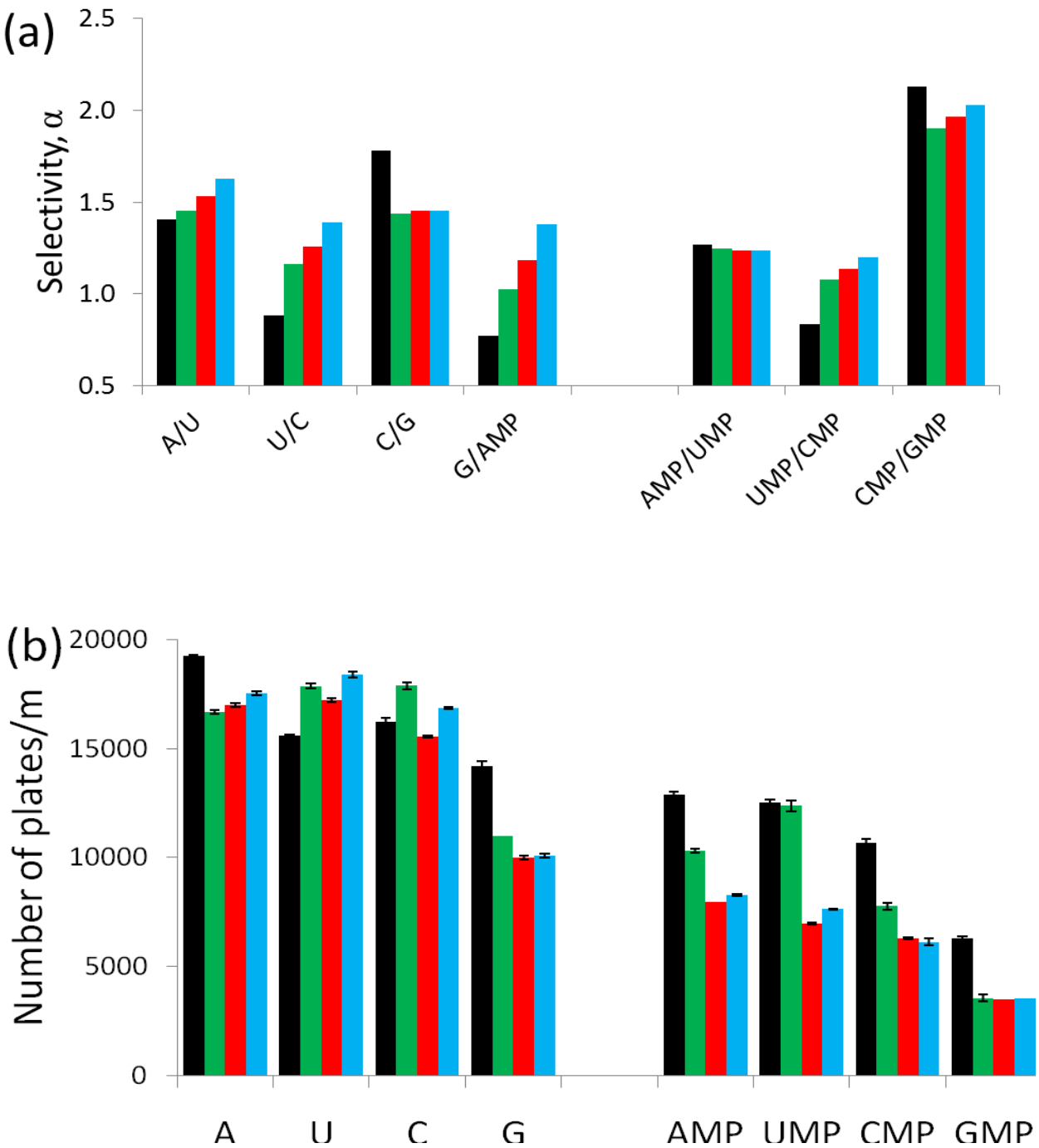

Figure 7: (a) Selectivity change with addition of CO_2 (b) Effect on the efficiency with addition of CO_2. Mobile phase contains (90:10) Methanol/ 75 mM ammonium acetate + 5mM DBN + 0.2M NaCl + 0 (■) 0.1 (■), 0.15 (■), 0.18 (■) mole fraction of CO_2.

the analytes capabilities for hydrogen bonding with the stationary phase or the water layer and cause, analytes with N-H or OH groups to have lower retention in methanol. Liu *et al* [3] explained this lack of retention by the fact that methanol is more polar than acetonitrile and has a strong ability of forming hydrogen bonds which might affect the formation of the water-enriched layer at the surface of the stationary phase. While the existence of the water layer in HILIC has been verified with acetonitrile as a weak eluent,[43] there is no evidence that it still exists with methanol and would be worth investigating.

However, in this study, we consider the addition of CO_2, which is a highly nonpolar modifier, and could be considered the weak eluent. We postulate that CO_2, might facilitate the formation of the water-enriched layer at the surface of the stationary phase, hence, increasing the partitioning of hydrophilic compounds.

Comparison to other HILIC separations

To the best of our knowledge, the separation of nucleosides/nucleotides by HILIC on an amide column with methanol as a weak eluent has not been previously reported; therefore, a direct comparison of our results to previously published data is not possible. However, HILIC studies on an amide column for nucleosides using acetonitrile as the weak eluent were reported and a difference in order of elution can be noticed. While both Guo *et al.*[29] and Marrubini *et al.* [44] observed U+A, C, G and U, A, C, G, we find the nucleotides eluting as A, C, U, G . A difference in order of elution when using methanol instead of acetonitrile has previously been reported[3, 4, 5]and can explain why our results differ from previous work on nucleosides.

Previous work in HILIC either focused on nucleosides or nucleotides[22, 23, 24] but typically not both at the same time. We formerly established that the separation of the nucleosides was faster and more efficient that previous isocratic separation including our previous EFLC study.[17] When comparing the separation of monophosphate nucleotides to other HILIC studies,[22, 23, 24] the resolution and selectivity obtained with HILIC-EFLC was either comparable or superior. The best and fastest separation of monophosphate nucleotides in HILIC was achieved by Zhou et al[24] in 7 minutes at 1ml/min. As shown in Figure 8, we, here, separate both the nucleosides and monophosphate nucleotides in just under 12 minutes at a similar flow rate. The use of EFLC allows very good separation of both nucleosides and monophosphate nucleotides, which is unprecedented in HILIC and very advantageous.

IV. Conclusions

Nucleosides and their monophosphates were successfully separated with good resolution and good peak capacity in only 16 minutes using an enhanced fluidity liquid mobile phase. This documents an expansion of elution window when using CO_2 in the mobile phase. The use of DBN to reduce band broadening proved to be valuable. Methanol/H_2O mobile phases are not commonly used in HILIC due to increased viscosity and lower selectivity compared to acetonitrile/H_2O. However, the addition of carbon dioxide lowers the viscosity and also enhances the selectivity.

This study showed that ternary mixture: CO_2/methanol/water provides an interesting alternative to acetonitrile/water as a mobile phase in HILIC: it presents the advantages of

methanol such as increased solubility of buffers and analytes, change in order of elution, reduced cost, eco-friendly waste without its drawbacks of insufficient retention and separation.

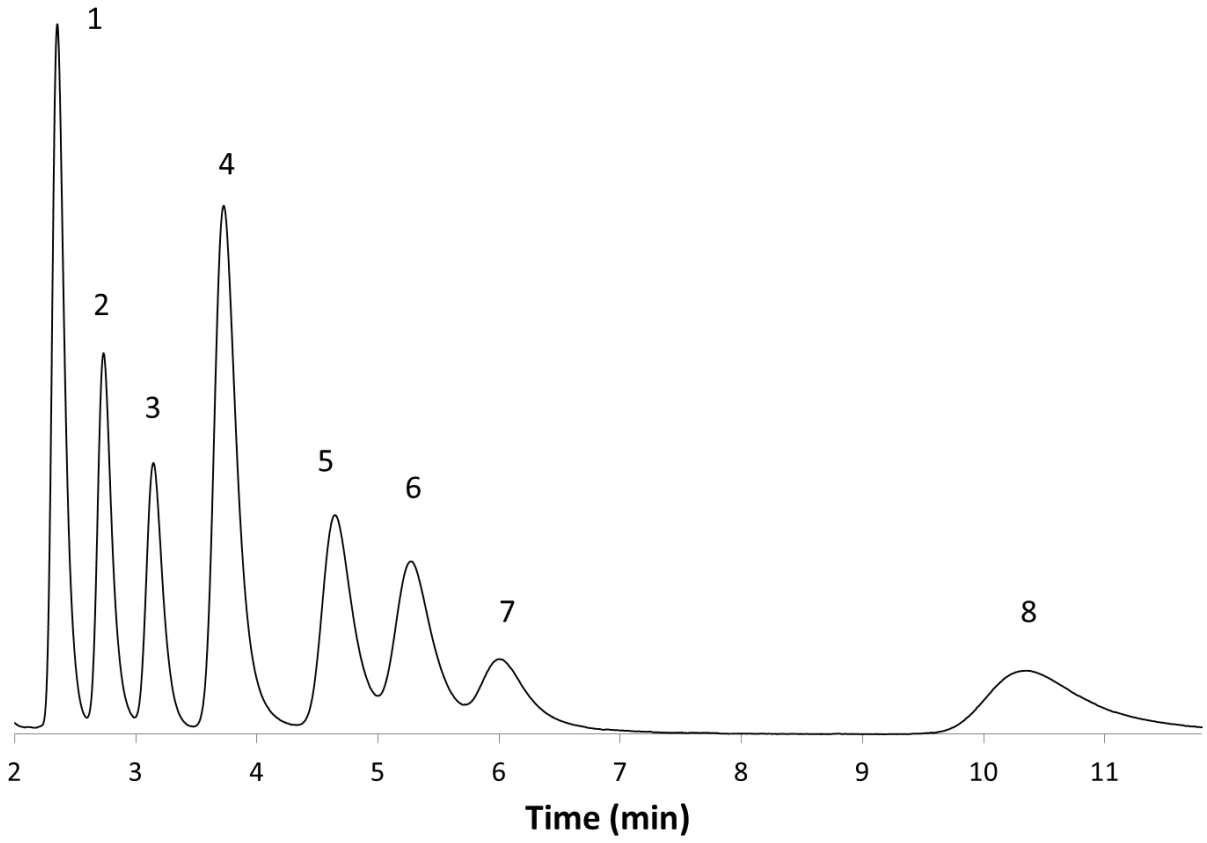

Figure 8: Separation of nucleosides and monophosphate nucleotides at 1mL/min. Conditions: (90:10) Methanol/ 75mM ammonium phosphate +5mM DBN +0.2 M salt and 0.18 mole fraction of CO_2. Adenosine (1), Cytidine (2), Uridine (3), Guanosine (4), AMP (5), CMP (6), UMP (7), GMP (8).

VI. References

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