Basic Properties of Novel Amino-Type HILIC Column and Separation of Hydrophilic Compounds

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Introduction

- Amino-type (NH_2) HILIC columns are widely utilized for analyzing hydrophilic compounds such as saccharides, metabolites, peptides, etc. However, it is well known that the chemical stability of NH_2 bonded phase columns is poor because of the presence of reactive Si-O bonds under aqueous mobile phase conditions.

- Various endcapping procedures have been proposed to reduce the impact of residual silanol groups and improve the chemical stability of C18 columns. Due to the reactivity of NH_2 groups it is not possible to perform the endcapping reaction after bonding the alkylamino ligand.

- Scientists at Tosoh Corporation developed a new NH_2-type HILIC column with the distinguishing feature that it is endcapped with trimethyl silane groups. By adopting the endcapping procedure, NH_2 groups can truly act as HILIC functional groups without immediate danger of column degradation due to hydrolysis of the silica backbone. The improvement in chemical stability over non-endcapped NH_2 columns was demonstrated by flushing the column with an aqueous/ACN eluent.

- In this poster we discuss the fundamental properties of this novel NH_2-type column and show applications of acidic and basic hydrophilic compounds such as saccharides, drug metabolites, etc. using this new NH_2-type column.

Experimental

- HPLC columns
  - Experimental NH_2-type HILIC column, 4.6mm ID x 15cm, Tosoh Corp.
  - TSKgel Amide-80, 3µm, 4.6mm ID x 15cm, Tosoh Corp.
  - TSKgel NH_2-60, 4.6mm ID x 25cm, Tosoh Corp.
  - Luna NH_2, 3µm, 4.6mm ID x 15cm, Phenomenex
  - Atlantis HILIC, 4.6mm ID x 15cm, Waters
  - ZIC-HILIC, 4.6mm ID x 15cm, Sequant
  - YMC Polyamine II, 4.6mm ID x 25cm, YMC
  - NH_2-504E, 4.6mm ID x 25cm, Showa Denko
  - Capcellpak NH_2, 4.6mm ID x 15cm, Shiseido
- All chemicals were purchased from Kishida Chemicals (Osaka).
- MTX samples were purchased from Schirck Laboratories (Jona, Switzerland).

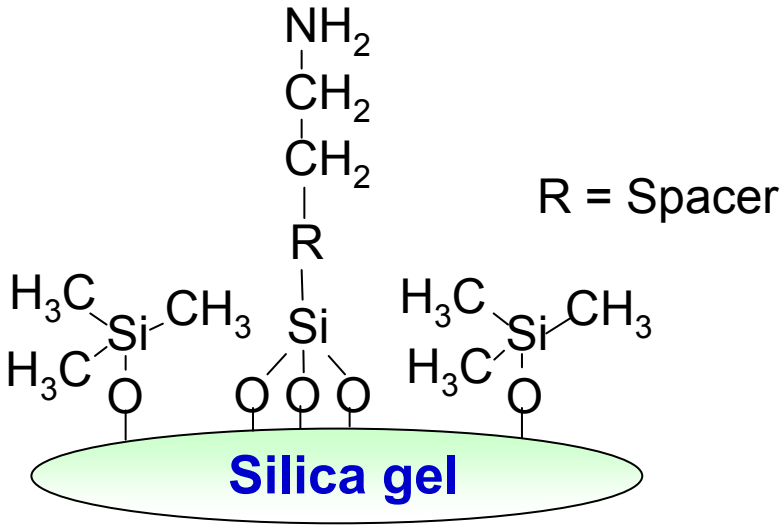
Basic Properties of Experimental NH_2-Type HILIC Packing

Properties	Experimental NH_2-Type HILIC Column
Base material	Silica gel
Mean particle size	3µm
Pore size (silica gel)	100Å
Surface area (silica gel)	450m^2/g
Functional group	Aminoethyl*
Endcapping	Trimethylsilyl group

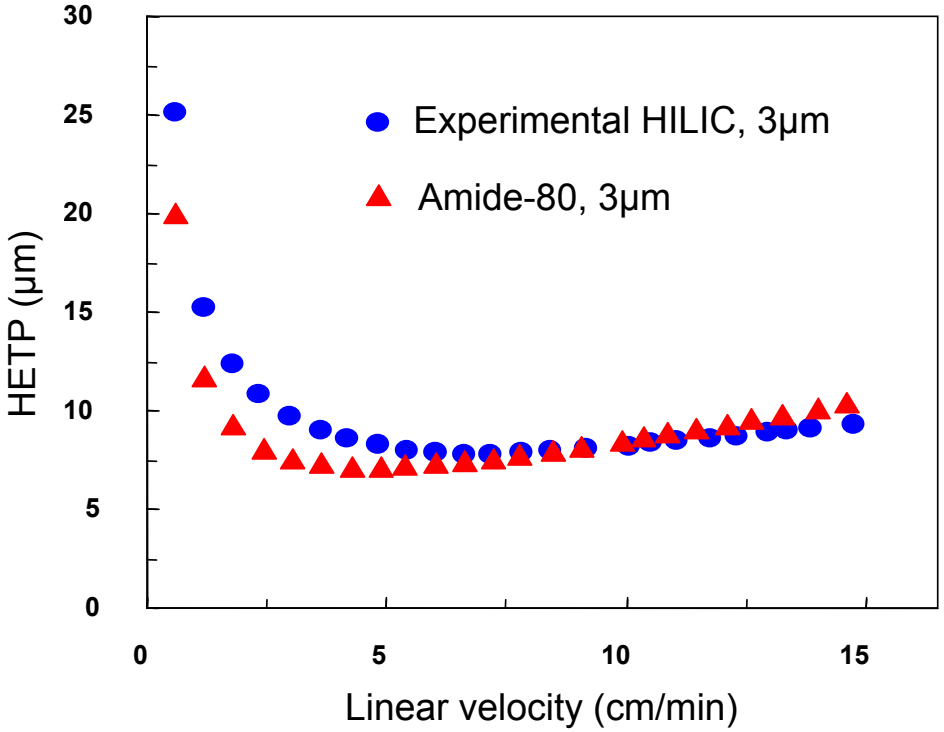
* Notes:

- The aminoethyl ligand is attached to the silica surface through an alkyl spacer.
- The nitrogen weight percentage of the NH_2-type HILIC column is approximately 2%, or about 20% higher than that of a TSKgel NH_2-60 column and approximately 20% lower than that of the TSKgel Amide-80 column.

Schematic Diagram of Experimental NH_2-Type HILIC Column



Van Deemter Curves for Commercial and Experimental HILIC Columns

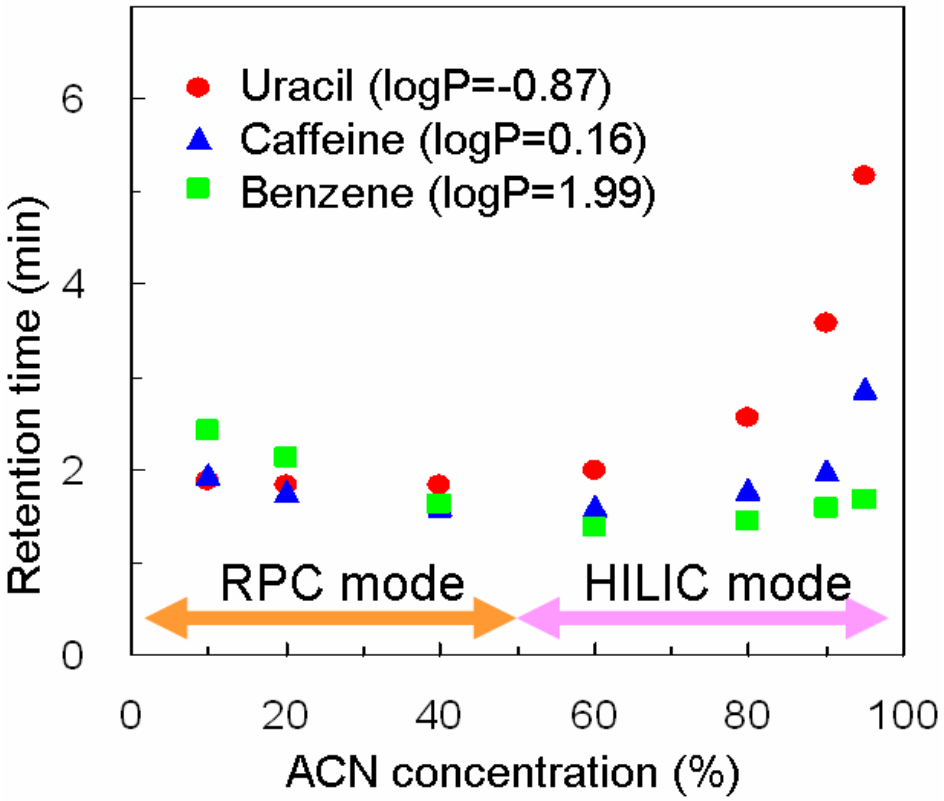


Columns: **Experimental NH_2 HILIC Column, 3µm, 4.6mm ID x 15cm**
TSKgel Amide-80, 3µm, 4.6mm ID x 15cm

Eluent: **H_2O/ACN=10/90**
Flow Rate: **0.1 ~ 2.4mL/min**
Detection: **UV@254nm**
Temp.: **40°C**
Inj. Volume: **10µL**
Sample: **uracil**

The optimal flow rate for the experimental NH_2-type HILIC, 3µm column is slightly higher than that for the TSKgel Amide-80, 3µm column. We speculate that this is due to the fact that the polymeric Amide-80 bonded phase hinders diffusion of solutes into and out of the pores.

Retention of Small Compounds under Various Acetonitrile Concentrations

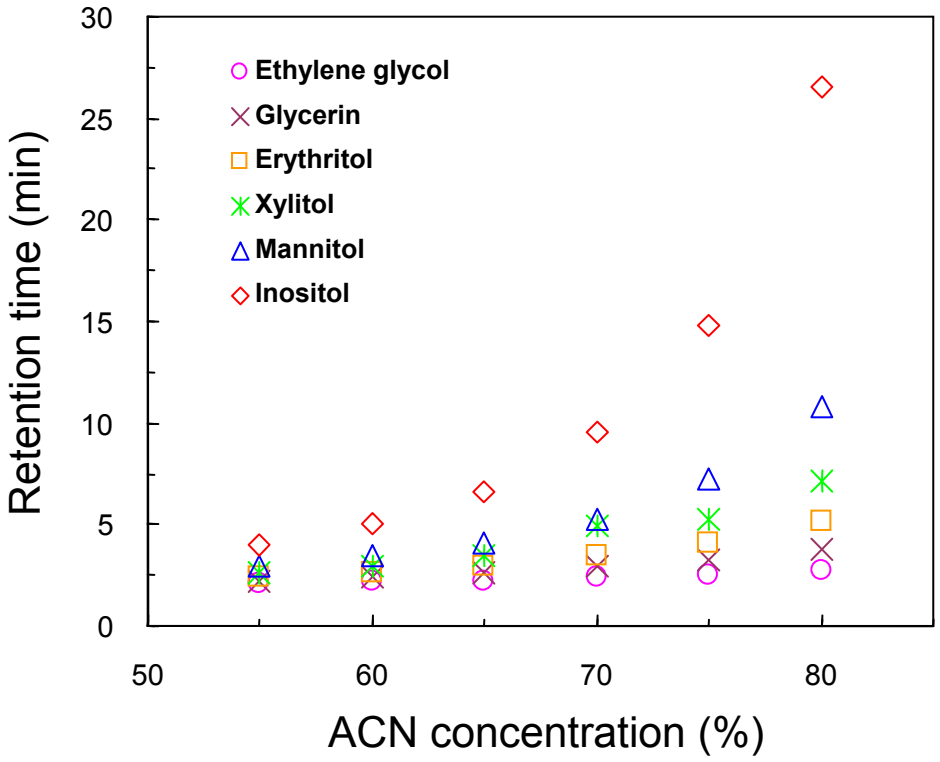


Column: Experimental NH_2 HILIC Column, 3µm, 4.6mm ID x 15cm

Eluent: 0.1% formic acid/ACN
Flow rate: 1.0mL/min
Detection: RI
Temperature: 40°C
Inj. Volume: 10µL
Sample: uracil, caffeine, benzene

In the HILIC mode, the retention times of uracil and caffeine increased with increasing percent ACN, while in the RPC mode, the more hydrophobic compound benzene is retained stronger at lower percent ACN. The change in separation mode takes place at about 50% acetonitrile.

Retention of Sugar Alcohols as Function of [ACN]

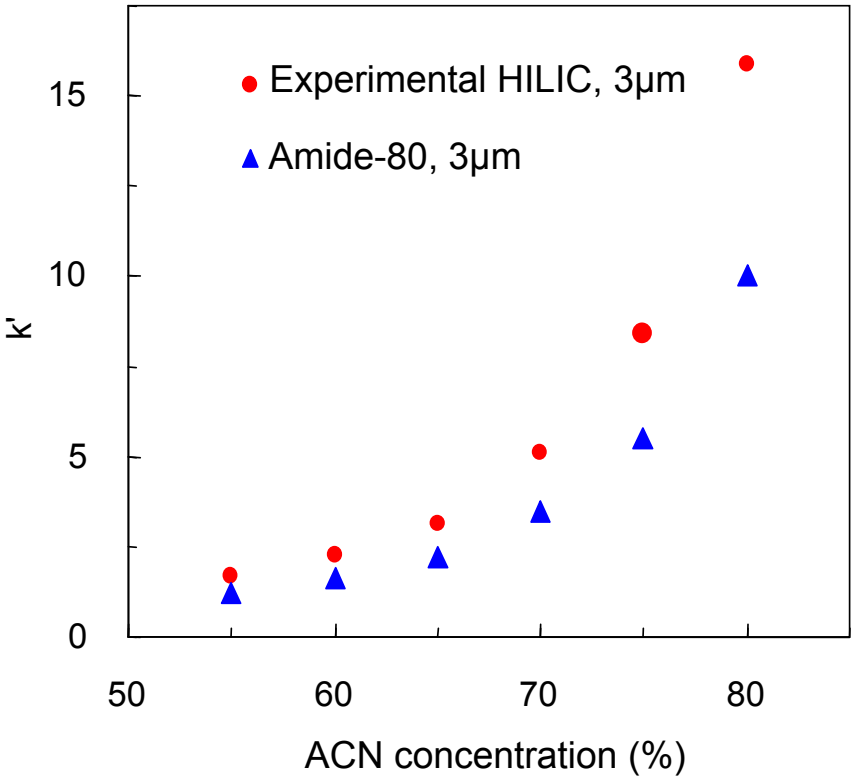


Column: Experimental NH_2 HILIC Column, 3µm, 4.6mm ID x 15cm

Eluent: H_2O/ACN=10/90
Flow rate: 1.0mL/min
Detection: RI
Temperature: 40°C
Inj. Volume: 10µL

The retention times of polyols are strongly affected by the ACN concentration. On the other hand, the retention times of ethylene glycol and glycerin are essentially the same at all ACN concentrations.

Retention of Inositol as Function [ACN]

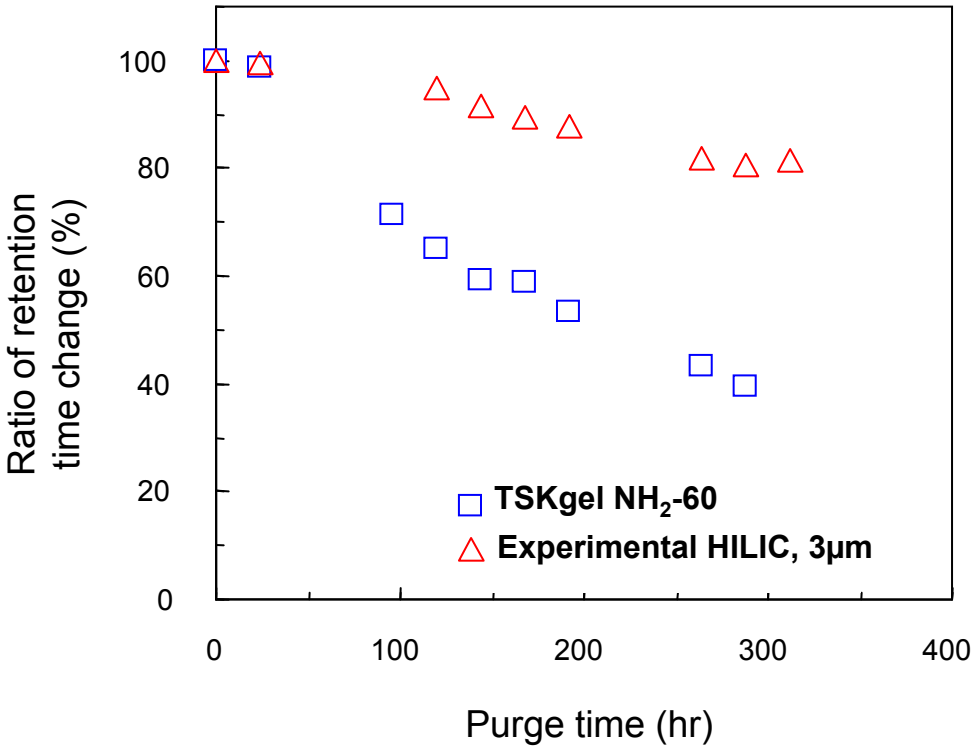


Columns: Experimental NH_2 HILIC Column, 3µm, 4.6mm ID x 15cm
TSKgel Amide-80, 3µm, 4.6mm ID x 15cm

Eluent: H_2O/ACN = 10/90
Flow rate: 1.0mL/min
Detection: RI
Temperature: 40°C
Inj. Volume: 10µL
Sample: inositol

Although retention of inositol increased with increasing percent ACN on both columns, inositol was stronger retained on the experimental NH_2-type HILIC column than on the TSKgel Amide-80 column.

Long Term Chemical Stability at H_2O/ACN = 10/90

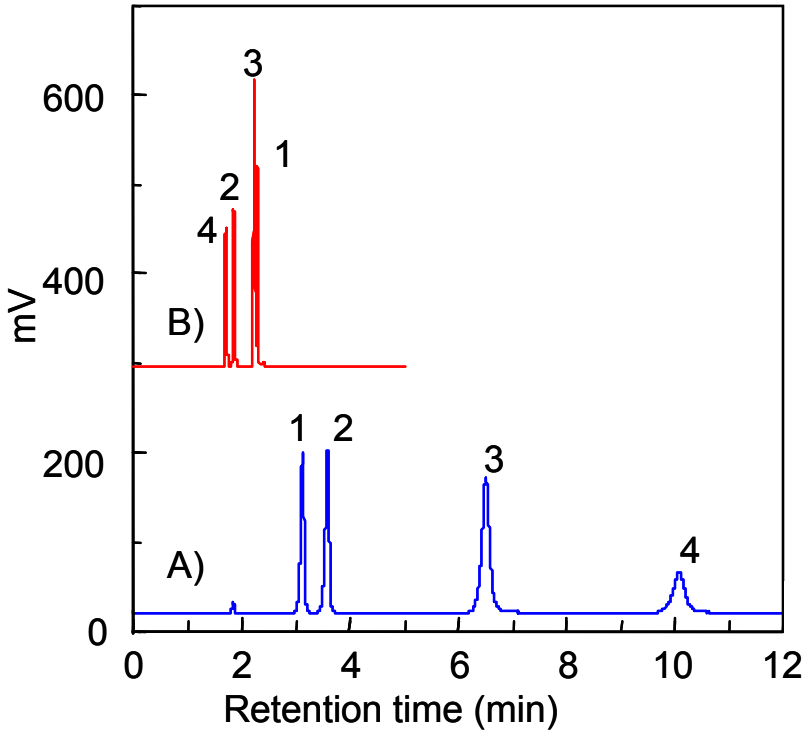


Conditions of Performance Check

Columns:	**Experimental NH_2 HILIC Column, 3µm, 4.6mm ID x 15cm**
	TSKgel NH_2-60, 4.6mm ID x 25cm
Eluent:	**H_2O/ACN = 25/75**
Flow rate:	**1.0mL/min**
Inj. Volume:	**10µL**
Temperature:	**40°C**
Detection:	**RI**
Sample:	**inositol**

After purging both columns for 300 hours, the retention time of inositol on the TSKgel NH_2-60 column decreased more than 60% from its initial retention time. In the case of the endcapped NH_2-type HILIC column, the retention time of inositol decreased less than 20%.

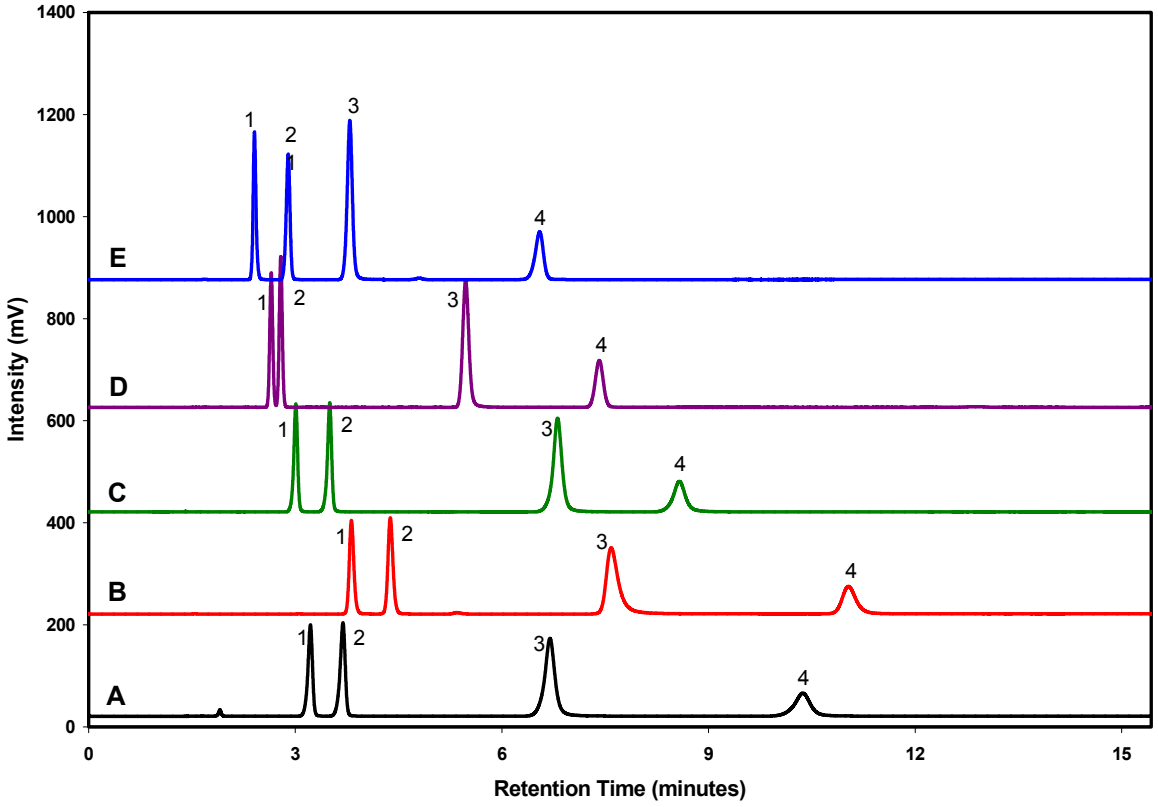
Comparison of Chromatograms of Nucleic Acid Bases, Part 1



Columns:	**A) Experimental NH_2 HILIC, 3µm, 4.6mm ID x15cm**
	B) TSKgel ODS-100V, 3µm, 4.6mm ID x 15cm
Eluent:	**A) 20mmol/L HCO_2NH_4/ACN=10/90**
	B) 20mmol/L HCO_2NH_4/ACN=90/10
Flow rate:	**1.0mL/min**
Injection vol.:	**10µL**
Temperature:	**40°C**
Detection:	**UV@254nm**
Sample:	**1. thymine**
	2. uracil
	3. adenine
	4. cytosine

The mobile phase conditions for the Reversed Phase and HILIC columns promote retention of the nucleic acid bases based on their hydrophobicity. It is clear, however, that another type of interaction dominates their retention on the NH_2 column, causing an almost complete reversal of elution order.

Comparison of Chromatograms of Nucleic Acid Bases, Part 2



Columns:

A: Experimental NH_2 HILIC Column, 3µm, 4.6mm ID x 15cm

B: TSKgel Amide-80, 3µm, 4.6mm ID x 15cm

C: Luna NH2, 3µm, 4.6mm ID x 15cm

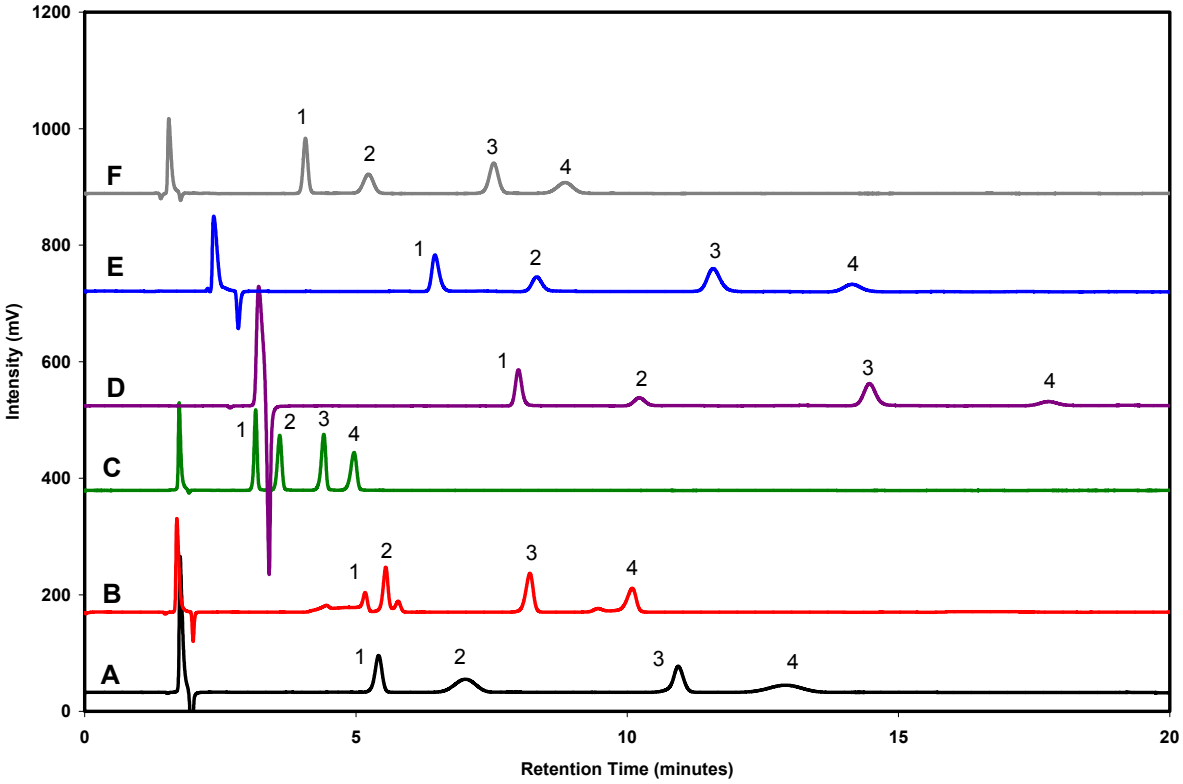
D: Atlantis HILIC, 4.6mm ID x 15cm

E: ZIC-HILIC, 4.6mm ID x 15cm

Eluent:	**20mmol/L ammonium formate/ACN = 10/90**
Flow rate:	**1.0mL/min**
Injection vol.:	**10µL**
Temperature:	**40°C**
Detection:	**UV@254nm**
Samples:	**1. thymine**
	2. uracil
	3. adenine
	4. cytosine

The four nucleic acid bases are retained longer on the TSKgel Amide-80 column than on any of the other HILIC columns.

Column Comparison for Mono- and Disaccharides



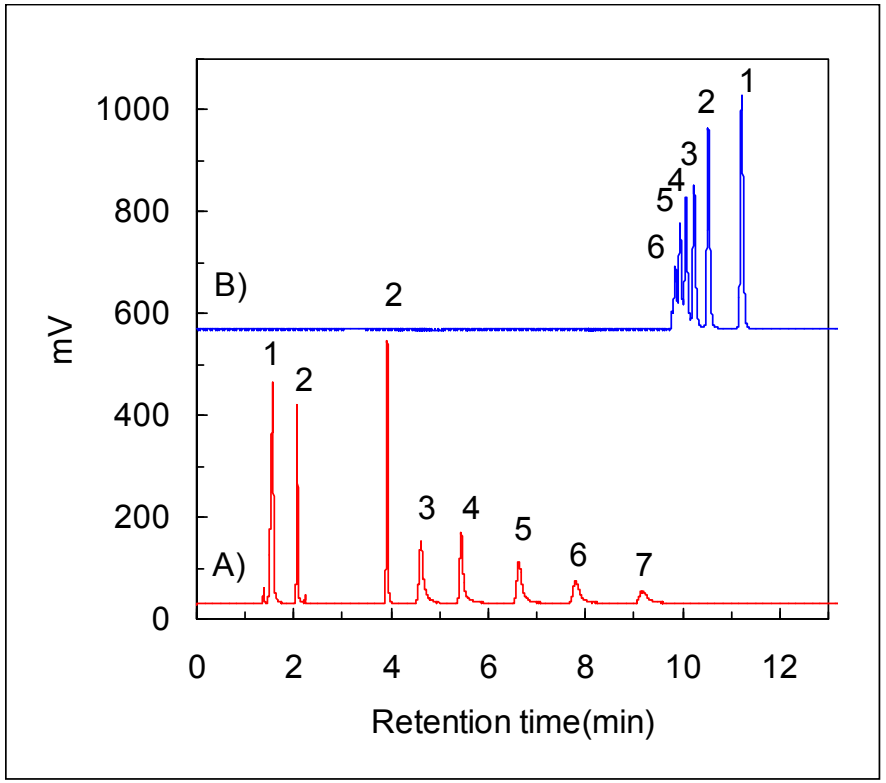
Columns:

A: Exp. NH_2 HILIC, 3µm, 4.6mm ID x15cm
B: TSKgel Amide-80, 3µm, 4.6mm ID x 15cm
C: Luna NH_2, 3µm, 4.6mm ID x 15cm
D: Polyamine, 4.6mm ID x 25cm
E: NH_2-50 4E, 4.6mm ID x 25cm
F: CAPCELLPAK NH_2, 4.6mm ID x 15cm

Eluent:	**H_2O/ACN = 25/75**
Flow rate:	**1.0 mL/min**
Inj. Volume:	**10µL**
Temperature:	**40°C**
Detection:	**RI**
Samples:	**1. fructose**
	2. glucose
	3. sucrose
	4. maltose

The experimental NH_2-type HILIC column shows the strongest retention for all sample components, when compensating for differences in column length.

Comparison of Chromatograms of MTX and its Derivatives



Columns:	**A) Experimental NH_2 HILIC, 3µm, 2.0mm ID x15cm**
	B) TSKgel ODS-100V, 3µm, 2.0mm ID x 15cm
Eluent:	**A: A) H_2O/ACN (10/90) + 0.1% TFA**
	B) H_2O + 0.1% TFA
	B: A) H_2O/ACN (10/90) + 0.1% TFA
	B) ACN + 0.1% TFA
Gradient:	**0% B (0min), 40% B (15min), 0% B (17min)**
Flow rate:	**0.20mL/min**
Injection vol.:	**10µL**
Temperature:	**40°C**
Detection:	**UV@313nm**
Samples:	**1. MTX (MTXPG) 2. $MTXPG_2$**
	3. $MTXPG_3$ 4. $MTXPG_4$
	5. $MTXPG_5$ 6. $MTXPG_6$
	7. $MTXPG_7$

Methotrexate and its derivatives ($MTXPG_{2\sim7}$) were separated on the experimental NH_2-type HILIC, 3µm and TSKgel ODS-100V, 3µm narrow bore columns. The MTX and polyglutamate derivatives were eluted in the order of the number of glutamate groups in their molecules on the NH_2-type HILIC column, but eluted in reverse order on the TSKgel ODS-100V column. Despite the early elution of MTX and $MTXPG_2$ on the NH_2-type HILIC column, the overall separation is better than what can be accomplished on the C18 column.

Conclusions

- In this study, a novel NH_2-type, 3µm HILIC column was developed and evaluated. The column contains Amino functional groups attached with an alkyl spacer and is fully endcapped.
- For several classes of hydrophilic compounds, the NH_2-type, 3µm HILIC column showed the strongest retention of several commercially available HILIC columns.
- As expected, hydrophilic compounds were retained by a reversed phase mechanism at low ACN concentrations, and by a HILIC mechanism at high ACN concentrations.
- Hydrophilic compounds show stronger retention on the NH_2-type, 3µm HILIC column than on the TSKgel Amide-80, 3µm column. We speculate that this is due to the higher ligand density and surface area of the NH_2-type, 3µm HILIC column.
- The column lifetime of the NH_2-type, 3µm HILIC column was improved three fold versus a TSKgel NH_2-60 column by using an endcapping reaction to inactivate residual silanol groups.
- The NH_2-type, 3µm HILIC column has the same functional group as the TSKgel NH_2-60 column, but has higher chemical durability due to endcapping. In general, amino-type columns are superior to amide-type columns for the separation of saccharides.
- ODS and HILIC columns comprise a powerful tool for separating hydrophilic compounds.