Stable amino-bonded HILIC column: An ideal tool for the separation of a wide variety of polar compounds

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Objectives

1. Show that silica-based TSKgel NH_2-100 columns are useful for the analysis of polar molecules using a conventional HPLC system.
2. Determine column lifetime for the endcapped amino-bonded phase that is employed in TSKgel NH_2-100 columns.
3. Demonstrate the simultaneous determination of a hydrophilic/acidic compound and a hydrophobic compound using a coupled column approach that combines a TSKgel NH_2-100 DC column and a TSKgel ODS-100V reversed phase column.

Introduction

- Reversed phase chromatography (RPC) is the most widely used mode of retention in HPLC.
- Very polar compounds are often not sufficiently retained in low percent organic, or even in 100% aqueous mobile phase.
- By using an amide or amino-bonded phase column, polar compounds can be retained by a normal phase or hydrophilic interaction chromatography (HILIC) retention mechanism using a mobile phase mixture of acetonitrile and water or buffer.
- In contrast to the retention behavior in reversed phase, in HILIC, solutes will be retained longer when increasing the percent acetonitrile.

Introduction

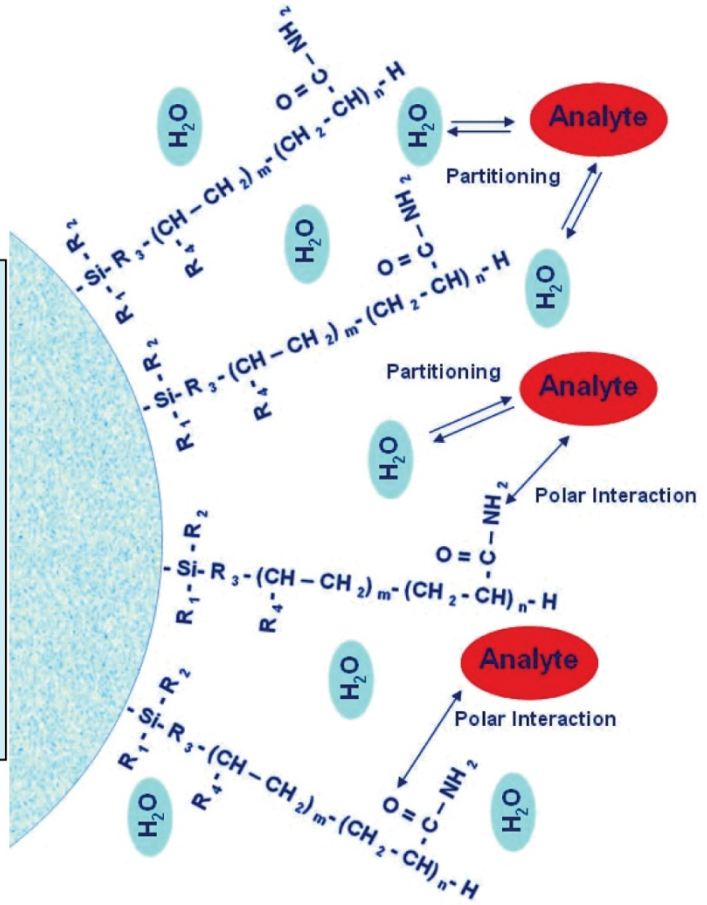
- Saccharides are fundamental substances that express various bioactivities and may exist independently or form complexes with proteins or lipids.
- Saccharides can be classified into monosaccharides, disaccharides, oligosaccharides, polysaccharides etc., based upon the degrees of polymerization and condensation.
- A polyol is an alcohol containing multiple hydroxyl groups. Sugar alcohols are a class of polyols.
- Sugar alcohols are commonly added to foods since they are of lower calorie content than the corresponding sugars.

Introduction

- Various analytical techniques have been used to analyze saccharides, including all modes of high performance liquid chromatography (HPLC).
- Normal phase chromatography, using amino-bonded phase columns, is an established technique for the analysis of saccharides, as it provides good selectivity with relatively short analysis times.
- Hydrophilic interaction liquid chromatography (HILIC) selectively retains saccharides and polyhydric alcohols, such as sugar alcohols. Monohydric alcohols and most of the substances with low polarity, however, elute in the void or very close to the void volume of the column.
- Conventional amino-bonded phase columns have limited column lifetime:
  - Reducing sugars can form glycosylamines with terminal primary amino groups.
  - Unprotected residual silanol groups do not prevent silica dissolution.

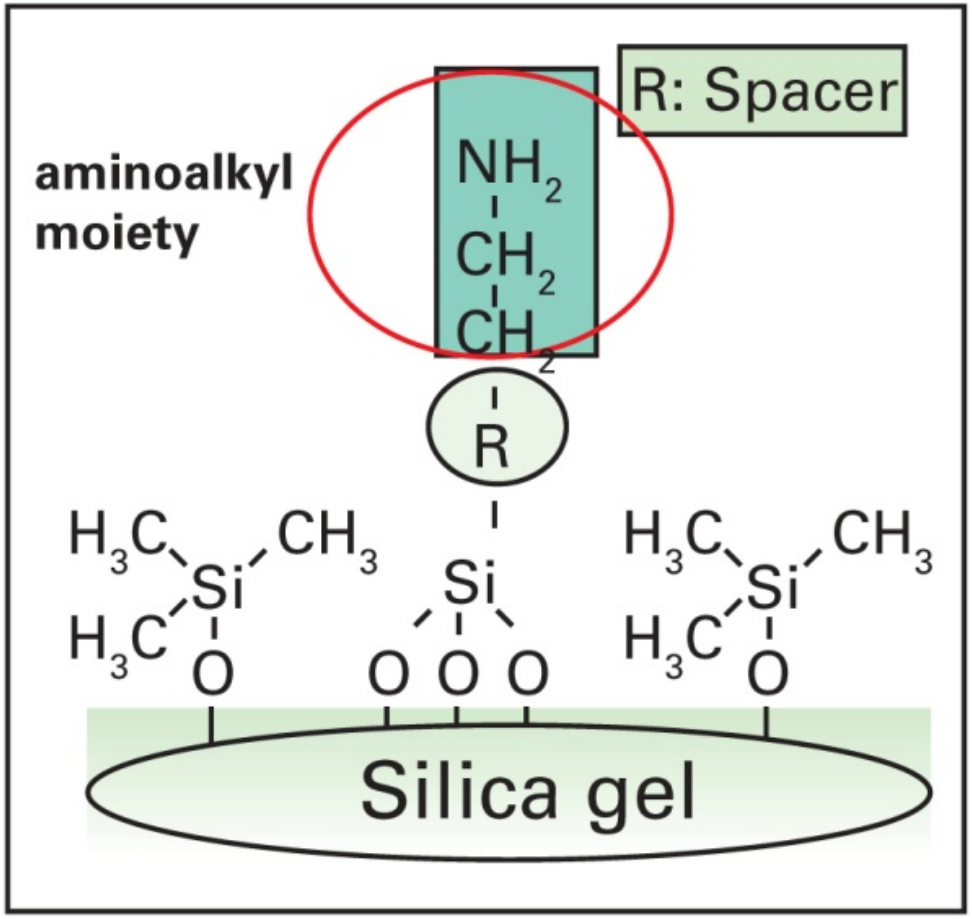
Structure and Mechanism

- Polar stationary phase as in normal phase LC
- Mobile phase similar to reversed phase (high organic)
- Elution in order of increasing hydrophylicity



Mechanism of Hydrophilic Interaction Liquid Chromatography (HILIC)

Schematic Diagram of Stationary Phase of TSKgel NH_2-100, 3µm



Properties of TSKgel NH_2-100, 3µm Packings

Base material	Silica
Particle size (nominal)	3µm
Pore size (nominal)	10nm
Specific surface area (nominal)	450m^2/g
Ligand *	Alkylamino
End-capping reagent	Trimethylsilyl groups

* Alkyl spacer also incorporates 2nd and 3rd amino groups

TSKgel NH_2-100 DC, 3µm Column and Its Direct Connection with Another Column

Column size	Theoretical plates	Asymmetry factor
4.6mm ID x 5cm	>= 6,000	0.90 - 1.30

Features of TSKgel NH_2-100 DC, 3µm Column

- Same packing material as in TSKgel NH_2-100 columns.
- Endfitting design allows direct connection to a standard TSKgel column, such as a TSKgel ODS-100V reversed phase column.
- Direct column connection reduces extra-column band broadening and thus provides optimal efficiency for the column set.
- The featured application demonstrates that a hydrophilic acidic compound can be retained on the TSKgel NH_2-100 DC column without the need to add an ion-pair reagent.

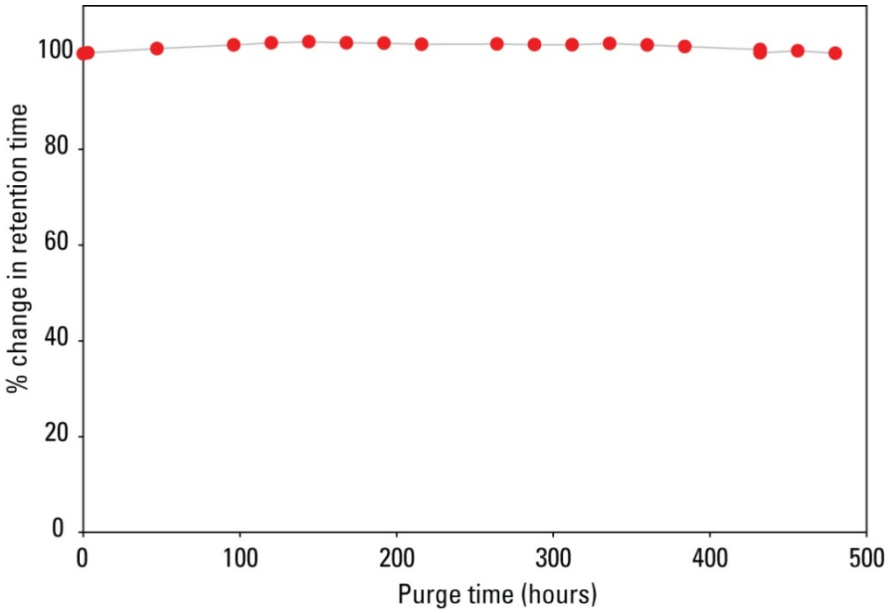
Materials and Methods

All analyses were carried out using an Agilent 1200 HPLC system run by Chemstation (ver B.04.01) unless mentioned otherwise.

Optimal chromatographic conditions (saccharides):

- Column: TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm
- Detection: RI
- Temperature: 50°C
- Flow rate: 0.2 mL/min
- Injection vol.: 2µL
- Mobile phase (Isocratic): 80% ACN in H_2O

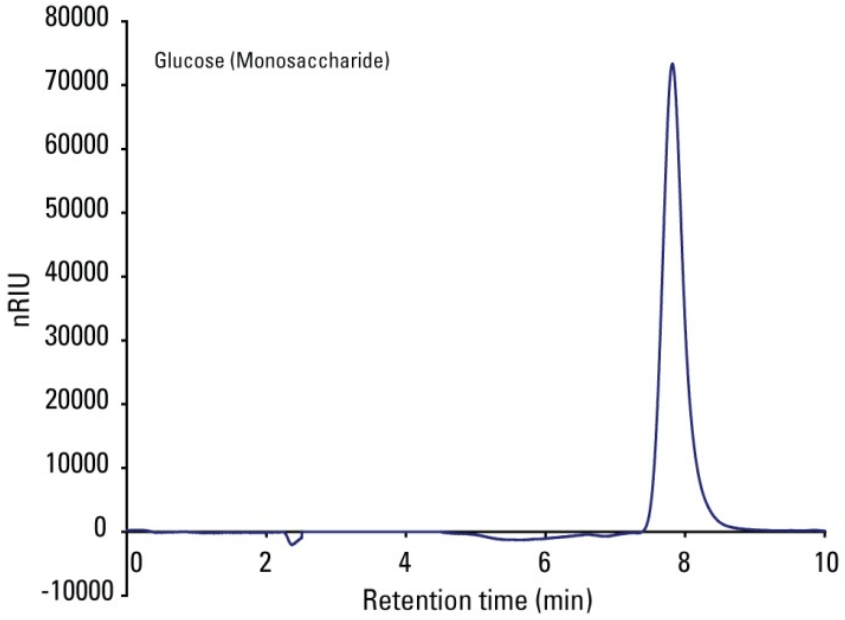
TSKgel NH_2-100 Column Stability



Column:	TSKgel NH_2-100, 3µm, 4.6mm ID x 5cm
Mobile phase:	H_20/ACN = 25/75
Flow rate:	1.0mL/min
Detection:	RI
Temperature:	40°C
Injection vol.:	10µL
Sample:	inositol

- **After flushing a TSKgel NH_2-100 column with 18L mobile phase (300 hours), retention of inositol showed minimal change.**
- **A column lifetime study using 5-fluoro uracil yielded about 1000 injections without change in the capacity factor (data not shown).**

Analysis of Glucose (monosaccharide) using a TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm Column



Columns:	TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm
Mobile phase:	80% ACN in H_2O
Flow rate:	0.2mL/min
Detection:	RI
Temperature:	50°C
Injection Vol.:	2µL

RT (min)	k	Area (mAU*S)	A_s	Plates (N)
7.822	11.4	1.59×10^6	1.25	3377

Limit of detection (LOD) of glucose – 100ppb

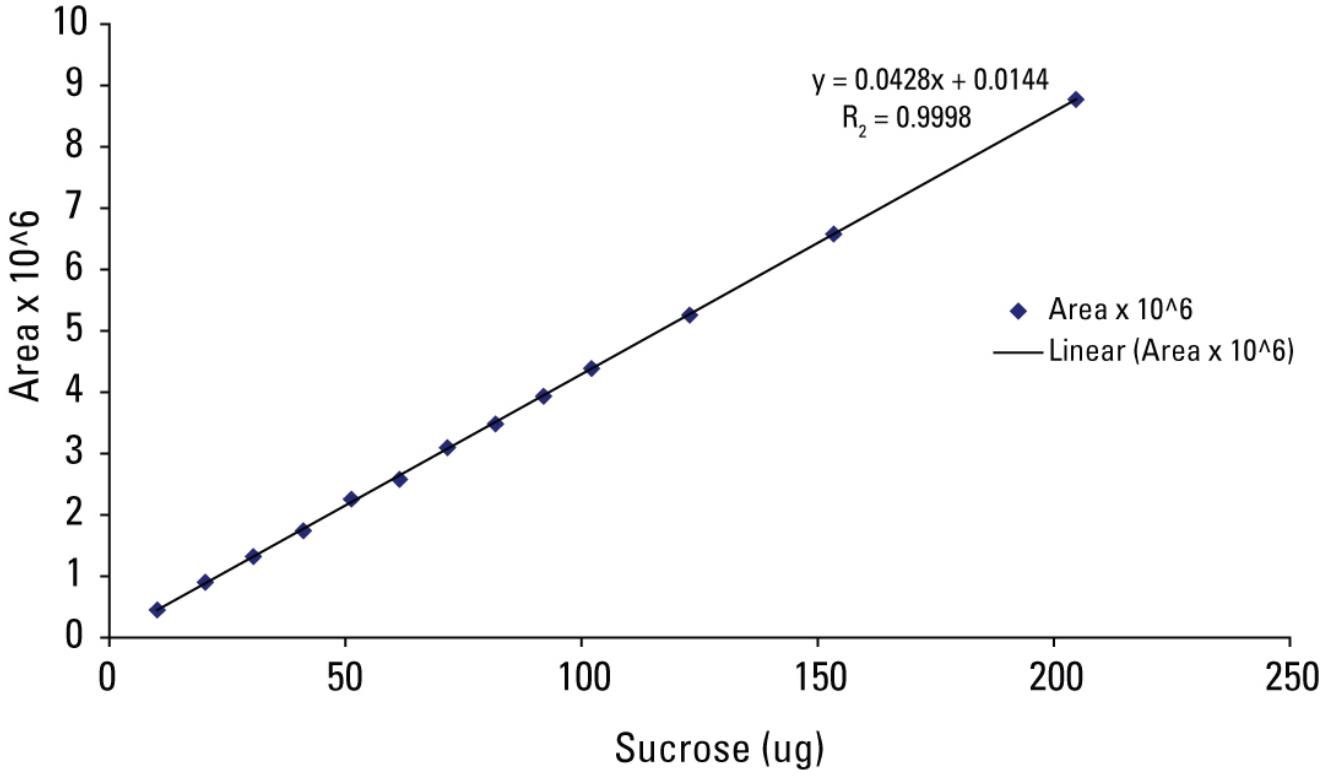
System Suitability Study

Sucrose

Run	RT (min)	k	Area (mAU*S)	A_s	Plates (N)
1	7.275	10.58	0.863×10^6	1.4	2732
2	7.28	10.59	1.07×10^6	1.4	2408
3	7.277	10.59	0.842×10^6	1.4	2734
Average	7.277	10.59	0.925×10^6	1.4	2624.6
Stdev	0.003	0.006	0.126×10^6	0.006	187.6
%RSD	0.000	0.000	0.136×10^6	0.008	0.071

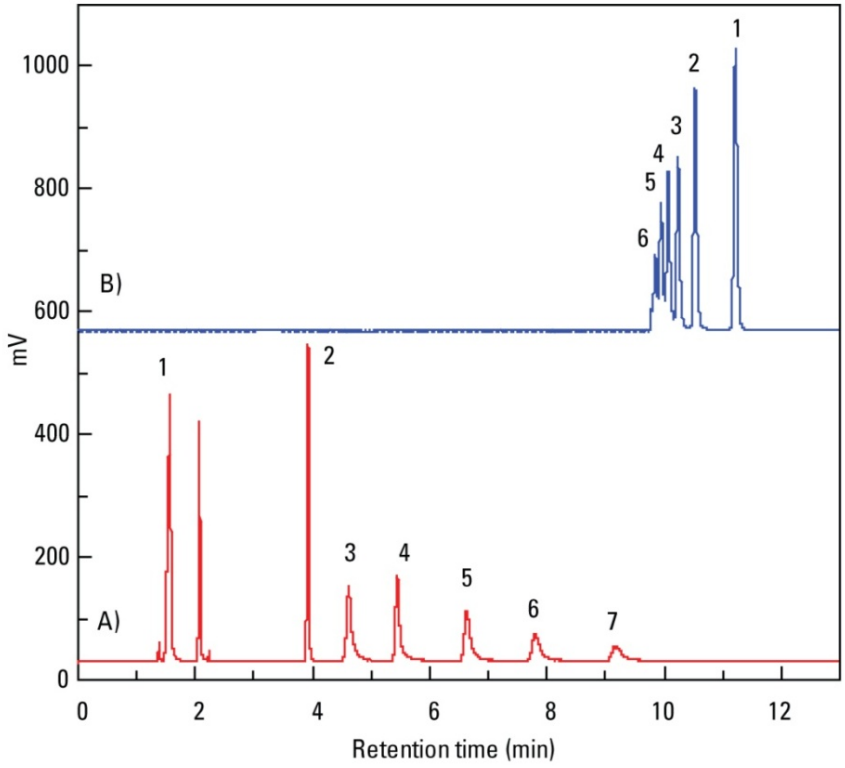
Three consecutive injections of sucrose yielded a very consistent results for all peak parameters that determine the suitability of the system and method.

Loading Capacity



Sucrose can be analyzed with a high degree of linearity over the experimental concentration range shown in this figure.

Comparison of Chromatograms of Methotrexate and Derivatives

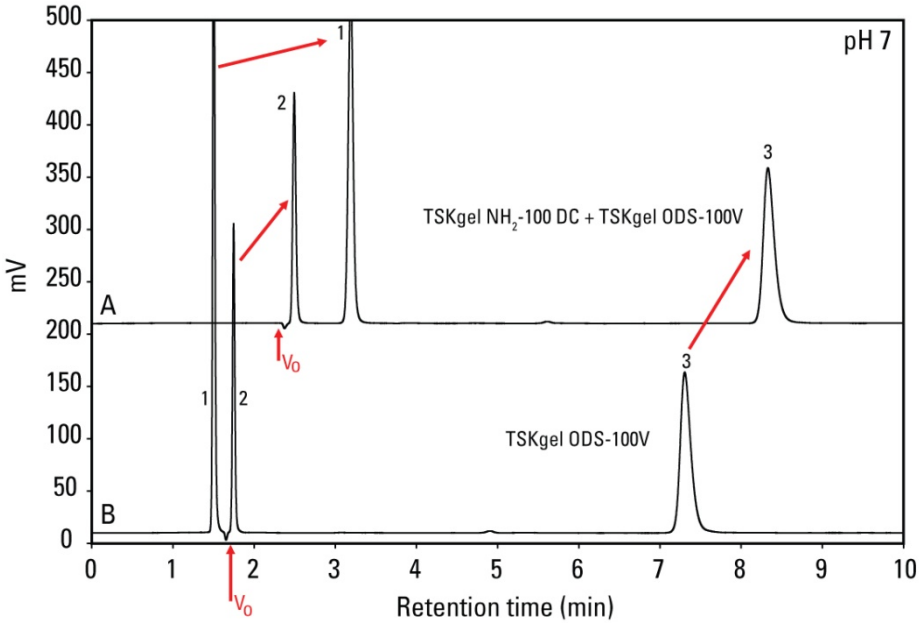


Columns:	A) TSKgel NH_2-100, 3µm, 2.0mm ID x 15cm
	B) TSKgel ODS-100V, 3µm, 2.0mm ID x 15cm
Mobile phase:	A: A) H_2O/ACN (10/90) + 0.1% TFA
	B) H_2O + 0.1% TFA
	B: A) H_2O/ACN (10/90) + 0.1% TFA
	B) ACN + 0.1% TFA
Gradient:	0% B (0min), 40% B (15min), 0% B (17min)
Flow rate:	0.20mL/min
Detection:	UV@313nm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

Analysis of a Basic Drug and Organic Acids by Coupling a HILIC and a Reversed Phase Column: (1) pH 7.0

- At pH 7.0, maleic acid and p-toluene sulfonic acid are negatively charged and are not retained on a C18 column in a 30% MeOH, 70% buffer eluent.
- After installing a TSKgel NH_2-100 DC, 3µm column prior to the TSKgel ODS-100V column, maleic acid is retained by the amino-bonded phase column through a weak anion exchange interaction.
- Although p-toluene sulfonic acid is a very strong acid and thus fully negatively charged at pH 7.0, it is not retained. This is possibly due to the influence of a high percentage of methanol.
- Desipramine, a secondary amine, is not retained on the amino column.
- Simultaneous determination of maleic acid and desipramine, in the presence of p-toluene sulfonic acid, was achieved on the coupled column system.

Analysis of a Basic Drug and Organic Acids by Coupling a HILIC and a Reversed Phase Column: (1) pH 7.0

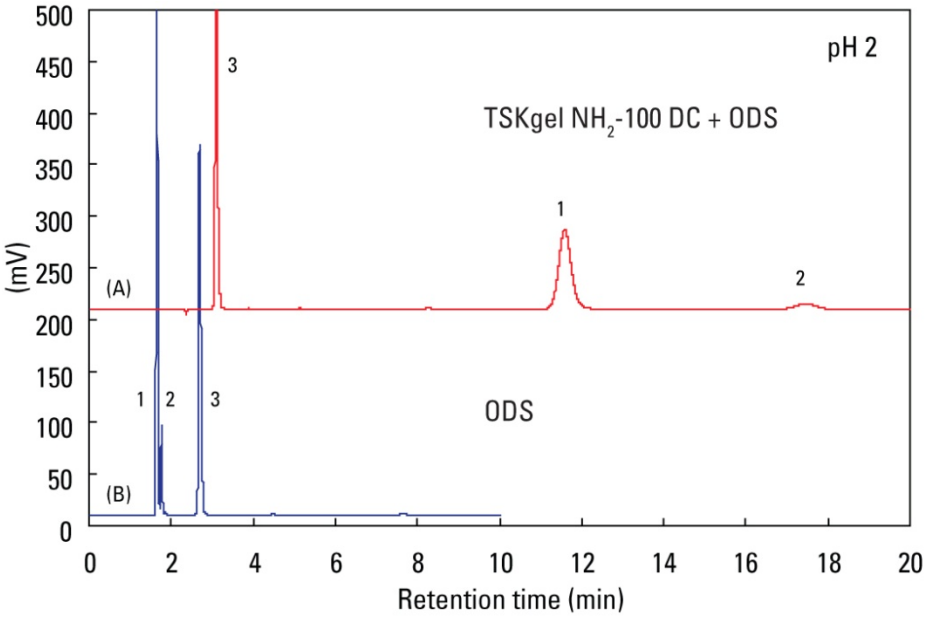


Columns:	A: TSKgel NH_2-100 DC, 3µm, 4.6mm ID x 5cm + TSKgel ODS-100V, 3µm, 4.6mm ID x 15cm B: TSKgel ODS-100V, 3µm, 4.6mm ID x 15cm
Mobile phase:	50mmol/L phosphate buffer, pH 7.0/MeOH = 30/70
Flow rate:	1.0mL/min
Inj. volume:	5µL
Temperature:	40°C
Detection:	UV@210nm
Samples:	1. maleic acid (50mg/L) 2. p-toluene sulfonic acid (50mg/L) 3. desipramine (50mg/L)

Analysis of a Basic Drug and Organic Acids by Coupling a HILIC and a Reversed Phase Column: (2) pH 2.0

- At pH 2, neither maleic acid or p-toluene sulfonic acid are retained on the TSKgel ODS-100V column. Desipramine is less retained than at pH 7.0 because it is fully dissociated.
- After installing a TSKgel NH_2-100 DC, 3µm column prior to the TSKgel ODS-100V column, both maleic acid and p-toluene sulfonic acid are more strongly retained due to anion exchange interaction with the fully dissociated, positively charged, amino-bonded phase.
- Note that desipramine, which is fully dissociated, elutes earlier than expected from the coupled columns as it is repulsed by the amino column.

Analysis of a Basic Drug and Organic Acids by Coupling a HILIC and a Reversed Phase Column: (2) pH 2.0

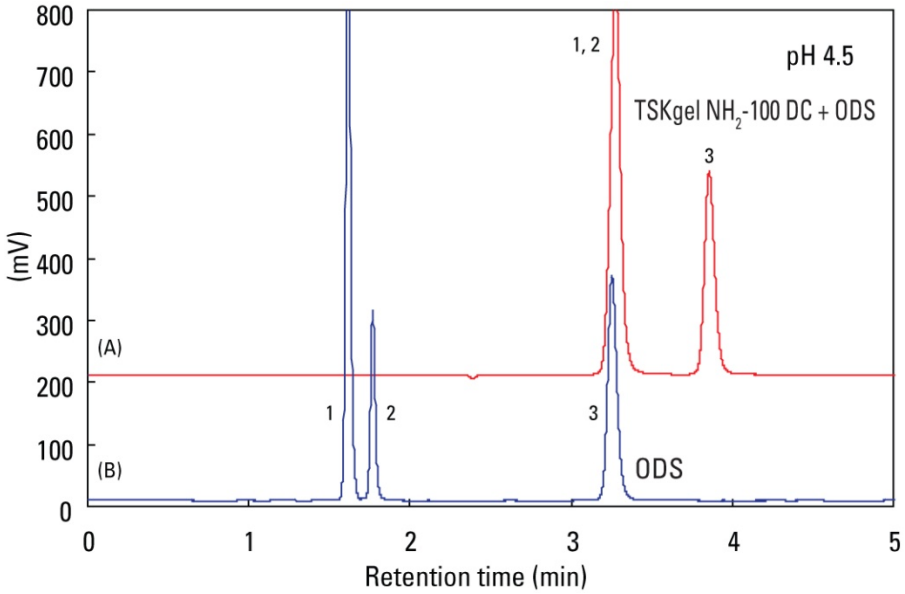


Column:	A) TSKgel NH_2-100 DC, 3µm, 4.6mm ID x 5cm + TSKgel ODS-100V, 3µm, 4.6mm ID x 15cm
	(B) TSKgel ODS-100V, 3µm, 4.6mm ID x 15cm
Mobile phase:	H_2O/MeOH/H_3PO_4 = 30/70/0.1, pH 2
Flow rate:	1.0mL/min
Detection:	UV@210 nm
Temperature:	40°C
Injection vol.:	5µL
Samples:	1. maleic acid (50mg/L)
	2. p-toluene sulfonic acid (50mg/L)
	3. desipramine (50mg/L)

Analysis of a Basic Drug and Organic Acids by Coupling a HILIC and a Reversed Phase Column: (3) pH 4.5

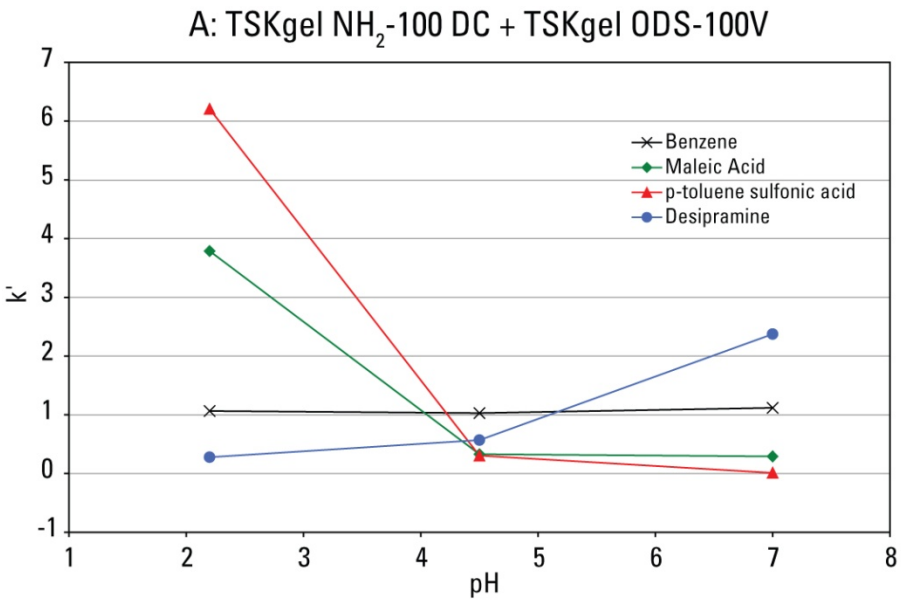
- At pH 4.5, both maleic acid and p-toluene sulfonic acid show substantial retention on the TSKgel NH2-100 DC, 3µm column but elute at the same retention time, while desipramine is retained longer.

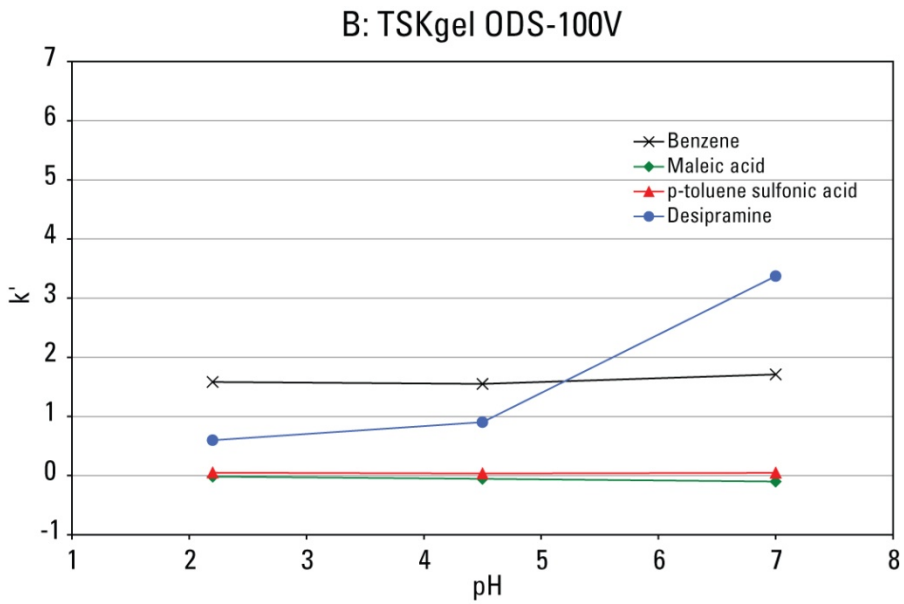
Analysis of a Basic Drug and Organic Acids by Coupling a HILIC and a Reversed Phase Column: (3) pH 4.5



Column:	(A) TSKgel NH_2-100 DC, 3µm, 4.6mm ID x 5cm + TSKgel ODS-100V, 3µm, 4.6mm ID x 15cm
	(B) TSKgel ODS-100V, 3µm, 4.6mm ID x 15cm
Mobile phase:	50mmol/L NaH_2PO_4, pH 4.5/MeOH = 30/70
Flow rate:	1.0mL/min
Detection:	UV@210 nm
Temperature:	40°C
Injection vol.:	5µL
Samples:	1. maleic acid (50mg/L)
	2. p-toluene sulfonic acid (50mg/L)
	3. desipramine (50mg/L)

Analysis of a Basic Drug and Organic Acids by Coupling a HILIC and a Reversed Phase Column: (4) Effect of pH





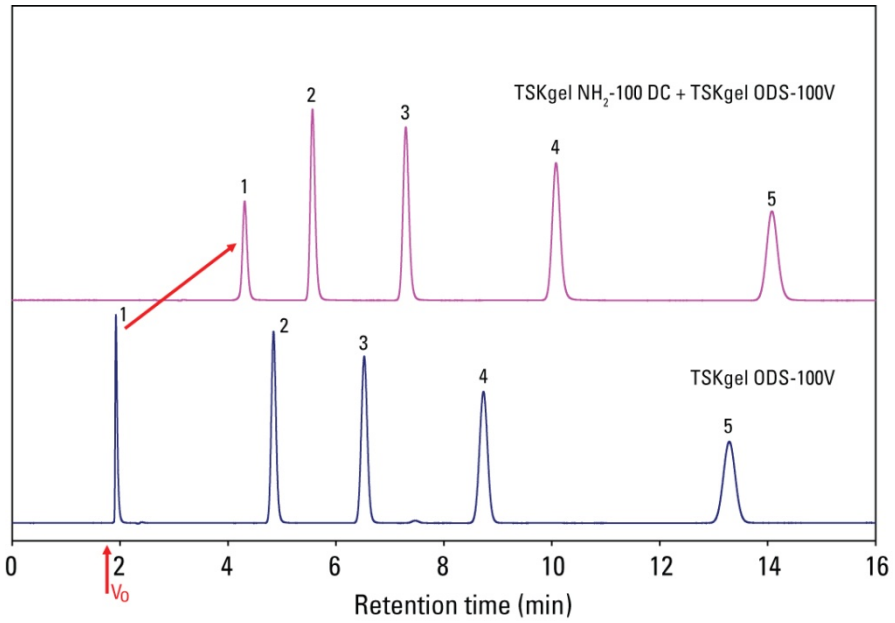
Column:	A: TSKgel NH_2-100 DC, 3µm, 4.6mm ID x 5 cm + TSKgel ODS-100V, 3µm, 4.6mm ID x 15 cm
	B: TSKgel ODS-100V, 3µm, 4.6mm ID x 15 cm
Mobile phase:	H_2O/MeOH/H_3PO_4 = 30/70/0.1
	50mmol/L NaH_2PO_4, pH 4.5/MeOH = 30/70
	50mmol/L phosphate buffer, pH 7.0/MeOH = 30/70

Separation of ingredients in cold medicine

Challenge: simultaneous determination of guaiacol sulfonic acid and other active pharmaceutical ingredients (API).

- Guaiacol sulfonic acid, a hydrophilic counter ion, is an expectorant used in pharmaceutical cold preparations that are sold over the counter (OTC) in many countries, but not in the US.
- Guaiacol sulfonic acid elutes in the solvent front on a C18 column, but is retained on a TSKgel NH_2-100 DC, 3um column.
- Direct Connection (DC) of the TSKgel NH_2-100 DC, 3µm column to a TSKgel ODS-100V, 3µm column allows for the simultaneous determination of APIs and guaiacol sulfonic acid in a single run.

Separation of ingredients in cold medicine



Columns:	A) TSKgel NH_2-100 DC, 3µm, 4.6mm ID x 5cm + TSKgel ODS-100V, 3µm, 4.6mm ID x 15cm B) TSKgel ODS-100V, 3µm, 4.6mm ID x 15cm
Mobile phase:	50mmol/L NaH_2PO_4, pH 2.5/MeOH = 65/35
Flow rate:	1.0mL/min
Inj. volume:	5µL
Temperature:	40°C
Detection:	UV@280nm
Samples:	1. guaiacol sulfonic acid (50mg/L) 2. anhydrous caffeine (25mg/L) 3. salicylamide (125mg/L) 4. aspirin (250mg/L) 5. ethenzamide (125mg/L)

Conclusions

- The TSKgel NH_2-100 HILIC column was successfully used for the separation of saccharides and methotraxate derivatives with good symmetry and efficiency.
- The calibration curve of sucrose shows high loading capacity with a high degree of linearity from 10 to 200µg injected on the column.
- A system suitability study for sucrose shows that the analyses could be reproduced with very low %RSD in peak parameters.
- The chemical and physical stability of the TSKgel NH_2-100 column was demonstrated by flushing the column with 30L mobile phase and injecting more than 1000 samples.
- Coupling the TSKgel NH_2-100 DC column to a reversed phase column allowed for the simultaneous determination of a hydrophobic basic compound in the presence of hydrophilic acidic compounds.
- TSKgel NH_2-100 columns expand the arsenal of high efficiency HILIC columns by providing retention through anion exchange in addition to retaining polar compounds through a normal phase retention mechanism.