Overview of the HILIC Mode of Chromatography for the Analysis of Polar Analytes

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Introduction

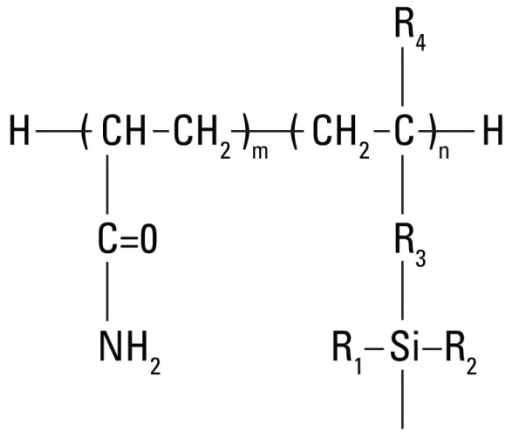
- Reversed Phase Chromatography (RPC) is the most widely used mode of retention in HPLC.
- Very polar compounds are often not sufficiently retained in low percent organic, or even in 100% aqueous mobile phase, in RPC mode.
- By using an amide- or amino-bonded phase column, polar compounds can be retained by a normal phase or hydrophilic interaction chromatography retention mechanism.
- Although non-polar organic mobile phases and a silica stationary phase were used traditionally in normal phase LC, today most normal phase separations are performed with aqueous-organic mobile phases and a more polar-bonded stationary phase.
- This mode of HPLC is now commonly referred to as HILIC, hydrophilic interaction chromatography.
- The order of elution in normal phase / HILIC is opposite that found in RPC for the same mixture of compounds.

Introduction

- TSKgel Amide-80 HILIC columns enable the analysis of labeled glycans, peptides, oligonucleic acid, and other hydrophilic small molecules.
- Packed with spherical silica particles covalently bonded with a carbamoyl moiety, the polar functional groups of the sample, such as hydroxy groups, form hydrogen bonds with the polar groups (amino groups) of the packing.
- A water-rich layer created in the bonded phase allows for partitioning of solutes with the more organic-rich mobile phase.
- The number of hydroxy groups, conformation and solubility in the mobile phase determine the order of elution.
- In addition to 10, 5 and 3 µm particle sizes, TSKgel Amide-80 columns are now available in 2 µm particle size.
- TSKgel Amide-80, 2 µm columns offer equivalent retention and selectivity as TSKgel Amide-80, 3 µm columns with higher resolution and a faster analysis time.
- An additional advantage of TSKgel Amide-80, 2 µm columns is that they retain more hydrophilic compounds than existing amide columns on the market.
- In this presentation we discuss applications using a 2 µm amide-bonded phase and amino-alkyl bonded phase column for the separation of a number of polar molecules.
- In addition, the effect of particle size on resolution and analysis time, retention and selectivity is discussed.

Introduction: TSKgel Amide-80 Columns

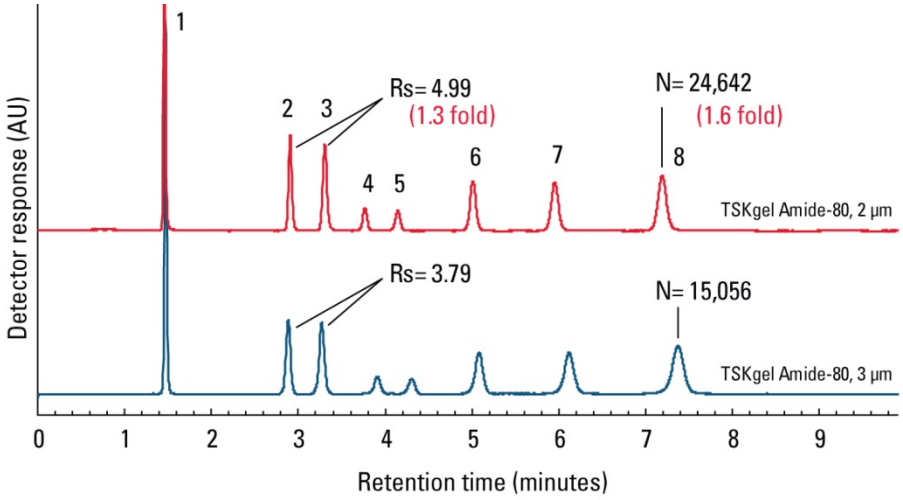
Structure:



Product attributes:

Pore size (silica):	8 nm
Particle size (mean):	2, 3, 5 or 10 µm
pH stability:	2.0 - 7.5
Functonal group:	Carbamoyl
Max. temperature (°C):	50 °C (2 & 3 µm), 80 °C (5 & 10 µm)
Surface area (m^2/g):	450

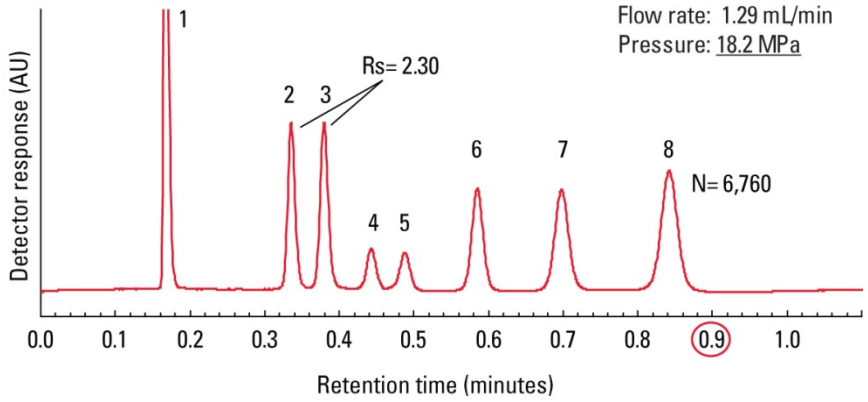
Comparison of Selectivity of 2 µm and 3 µm TSKgel Amide-80 Columns



Columns:	TSKgel Amide-80, 2µm, 3.0 mm ID × 15 cm TSKgel Amide-80, 3µm, 3.0 mm ID × 15 cm
Mobile phase:	20 mmol/L NH_4OAc, pH 4.7 / acetonitrile = 10/90
Flow rate:	0.43 mL/min
Detection:	UV @ 254 nm
Temperature:	40 ˚C
Injection vol.:	2 µL
Samples:	1. toluene (1 g/L) 2. theophylline (0.1 g/L) 3. theobromine (0.1 g/L) 4. NPβGlu (0.1 g/L) 5. NPαGlu (0.1 g/L) 6. 2'-deoxyuridine (0.1 g/L) 7. 5-methyluridine (0.1 g/L) 8. uridine (0.1 g/L)

- A set of hydrophilic molecules, such as nucleosides, sugars, hydrotropes, etc. were analyzed using TSKgel Amide-80, 3.0 mm ID × 15 cm columns of 2 and 3 µm particle size.
- As seen above, similar chromatographic profiles were obtained with similar selectivity.
- The smaller particle size of the TSKgel Amide-80, 2 µm column yielded a 1.6-fold increase in theoretical plates and a 1.3-fold higher resolution.

Ultra-high Fast Separation with TSKgel Amide-80, 2 µm Column



Column:	TSKgel Amide-80, 2 µm, 3.0 mm ID × 5 cm
Mobile phase:	20 mmol/L NH_4OAc, pH 4.7 / acetonitrile = 10/90
Flow rate:	1.29 mL/min
Detection:	UV @ 254 nm
Temperature:	40 °C
Injection vol.:	2 µL
Samples:	1. toluene (1 g/L)
	2. theophylline (0.1 g/L)
	3. theobromine (0.1 g/L)
	4. NPβGlu (0.1 g/L)
	5. NPαGlu (0.1 g/L)
	6. 2'-deoxyuridine (0.1 g/L)
	7. 5-methyluridine (0.1 g/L)
	8. uridine (0.1 g/L)

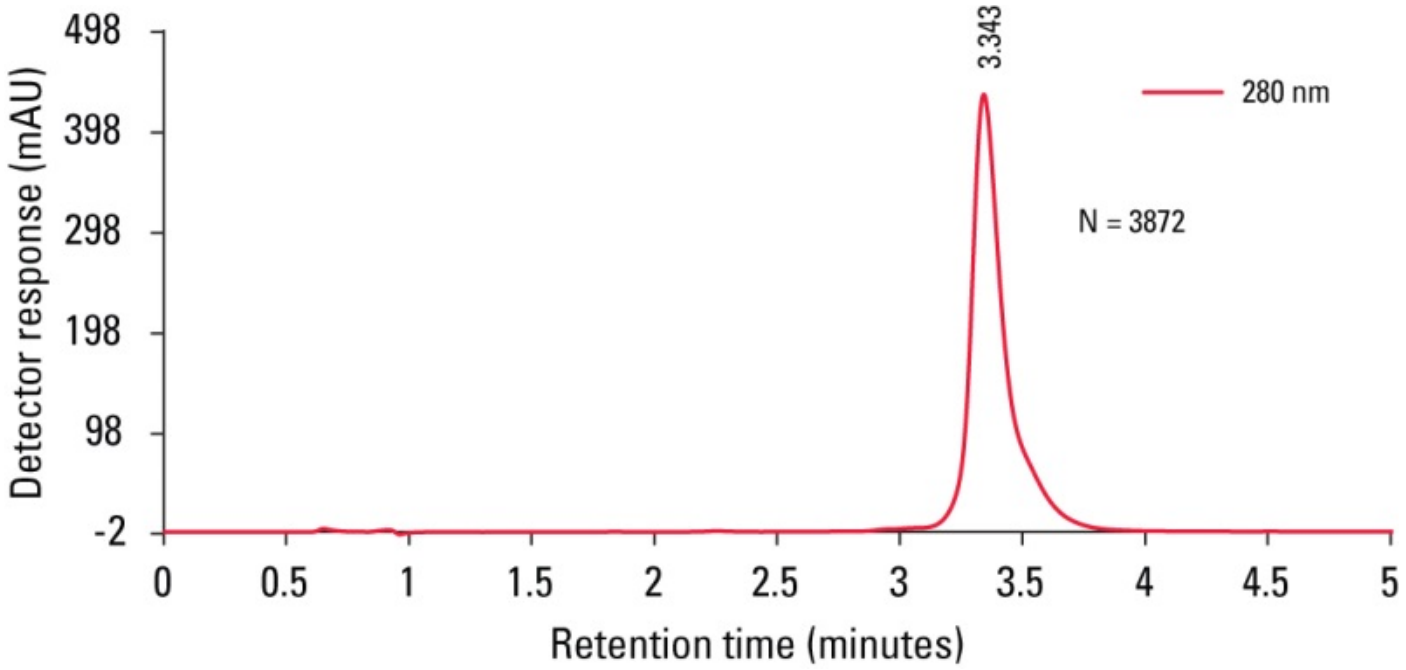
- A TSKgel Amide-80, 2 µm column showed impressive results for an ultra-high speed analysis of these same hydrophilic molecules.
- A less than one minute separation was obtained using a TSKgel Amide-80, 2 µm column at a flow rate of 1.29 mL/min.
- In addition, the 2 µm column showed a lower pressure drop than the maximum pressure of a conventional HPLC system.
- Therefore, it is not necessary to use a UHPLC system for this type of ultra-high fast separation.

Analysis of 5-fluorocytosine using the TSKgel Amide-80, 2 µm Column

- Uridine is a glycosylated pyrimidine-analog containing uracil attached to a ribose ring (or more specifically, a ribofuranose) via a β-N1-glycosidic bond.
- Uridine and its derivatives are in natural foods, such as beets, and have shown to prevent depression in rats as effectively as antidepressant drugs. *(Ref: Biol Psychiatry 57 (4): 343–50)*
- Uridine is a naturally occurring pyrimidine derivative. It is slightly basic in nature and hydrophilic.
- 5-fluorocytosine (5FC) is an oral antifungal. It is a hydrophilic molecule of relatively low molecular weight.
- Vitamin B12 is a hydrophilic water-soluble vitamin, essential for neurological function, erythropoiesis, enzymes synthesis, and DNA/RNA formation.
- Below we show the separation of these three polar compounds using a TSKgel Amide-80, 2 µm column under the following chromatographic conditions:

Mobile phase:	70% acetonitrile: 30% 25 mmol/L phosphate buffer, pH 2.52
Flow rate:	0.35 mL/min
Temperature:	25 ˚C
Injection vol.:	5 µL
Sample concentration:	1.0 g/L

Analysis of Vitamin B12 using the TSKgel Amide-80, 2 µm Column



Preliminary results show that a 2 µm, TSKgel Amide-80 column separates vitamin B12, a water-soluble vitamin. The study of the effect of the temperature on the peak shape and the separation efficiency is under progress.

Analysis of 5-fluorocytosine and 5-fluoro-2’deoxyuridine using the TSKgel Amide-80, 2 µm Column

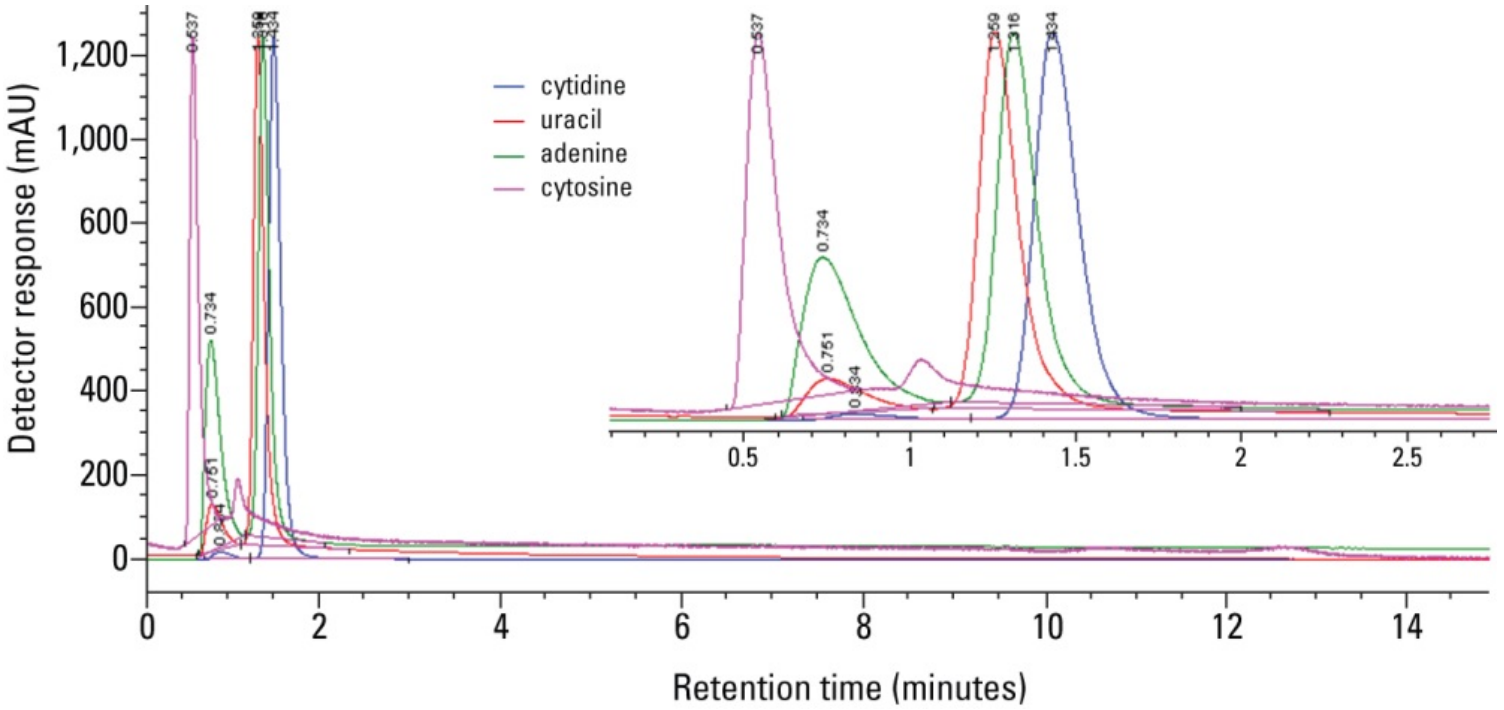
Sample	RT (min)	N (number of theoretical plates)
5-fluorocytosine	2.017	2087
5-fluoro-2’deoxyuridin	1.233	1620

Separation of Nucleobases in HILIC Mode using a HILIC Column and a Diol Coated SEC Column

- Nucleobases are highly hydrophilic in nature.
- Separation of four nucleobases in HILIC mode was tried under the following chromatographic conditions using a HILIC column and a Diol coated size exclusion chromatography (SEC) column under the following chromatographic conditions:

Instrumentation:	Agilent 1100 HPLC system run by Chemstation (ver B.04.02)
Columns:	TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm TSKgel Amide-80, 5 µm, 2.0 mm ID × 10 cm
Mobile phase:	A: acetonitrile (HILIC mode) B: 15 mmol/L ammonium bicarbonate, pH 7.4 (HILIC mode)
Mobile phase:	100 mmol/L phosphate/100 mmol/L sodium sulfate, pH 6.7 + 0.05% NaN_3 (SEC mode)
Gradient:	Isocratic
Flow rate:	0.4 mL/min
Detection:	UV @ 280 nm
Injection vol.:	1 µL
Temperature:	ambient
Samples:	uracil (1.5 mg/mL), adenine (1.5 mg/mL), cytosine (1.5 mg/mL), cytidine (1.5 mg/mL) from Sigma Aldrich

Separation of Nucleobases using the TSKgel Amide-80 HILIC Column



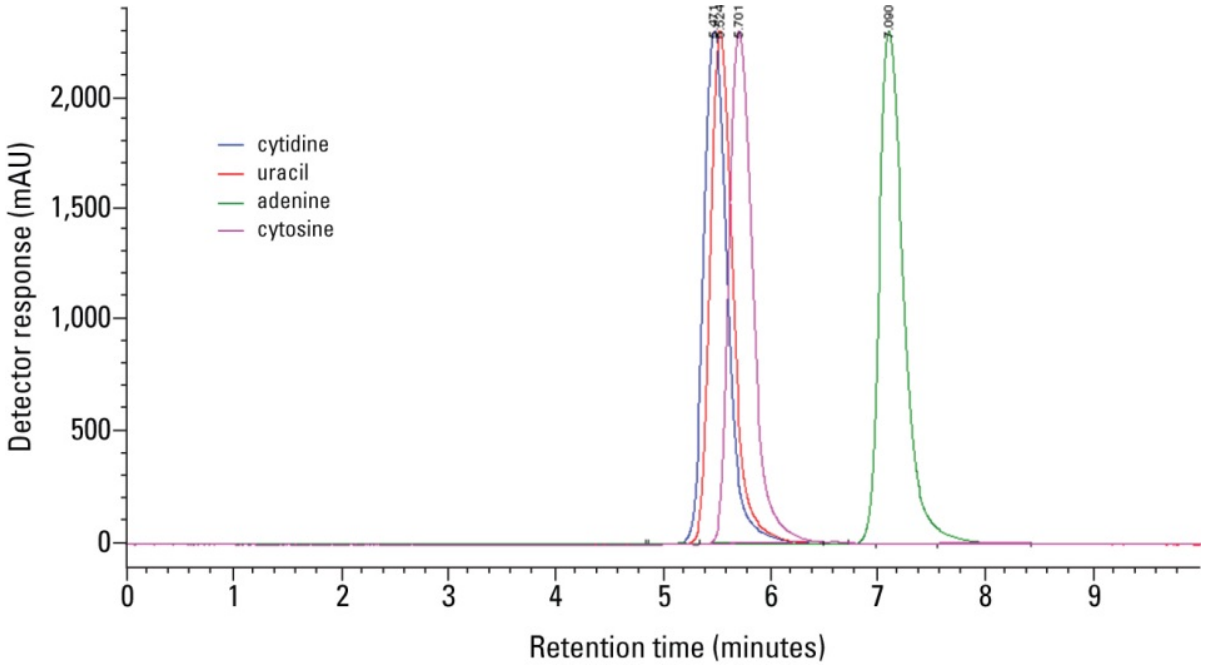
A TSKgel Amide-80 column under the above mentioned chromatographic HILIC conditions yielded poor separation of the four nucleobases with virtually no retention of any of the components.

Separation of Nucleobases using TSKgel SuperSW mAb HTP SEC Column under Conventional SEC Conditions and in HILIC Mode

Following is the product attributes of the TSKgel SuperSW mAb HTP SEC column, the surface of which has been shielded from interacting with proteins by derivatization with ligands containing diol functional groups. This column was used in HILIC mode for the separation of nucleobases.

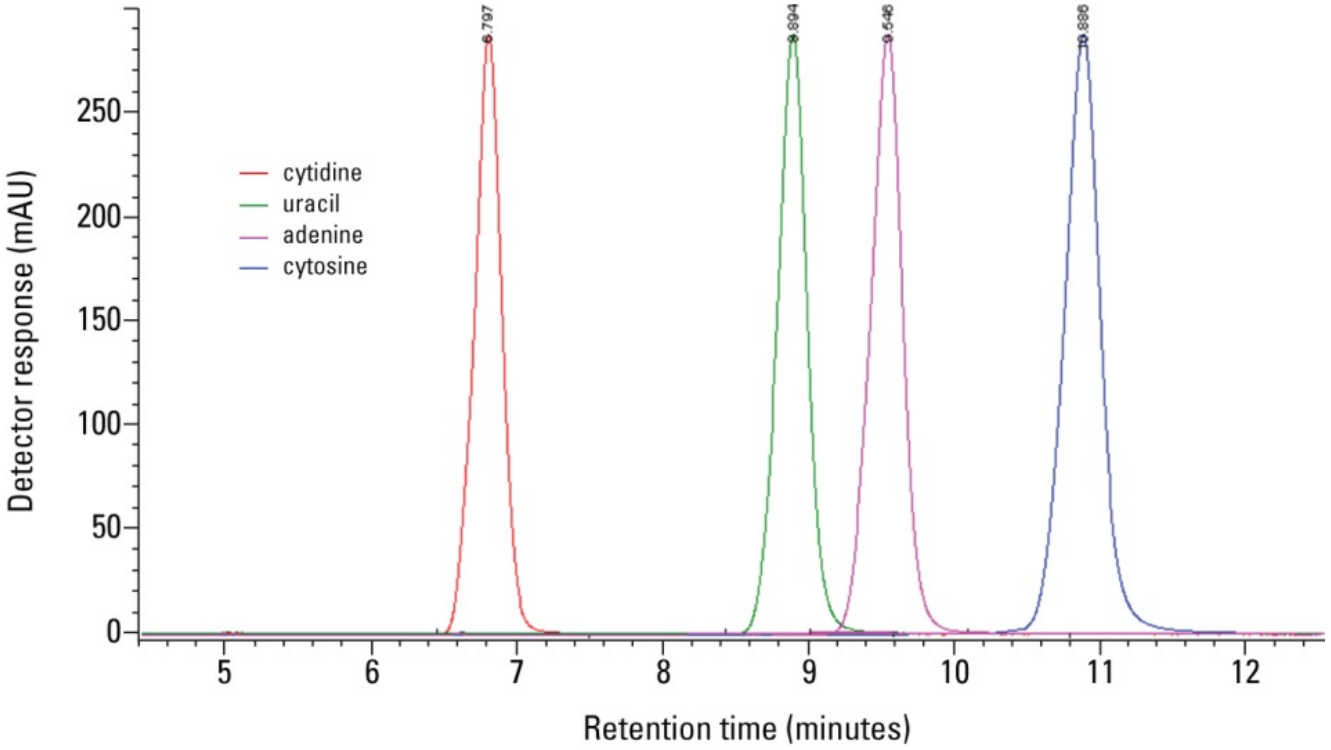
Base material:	25 nm
Particle size (mean):	4 µm
Pore size (mean):	25 nm
Functonal group:	Diol
pH satability:	2.5 - 7.5
Calibration range:	10,000 - 500,000 Da (globular proteins)

Separation of Nucleobases using the TSKgel SuperSW mAb HTP SEC Column under Conventional SEC Conditions



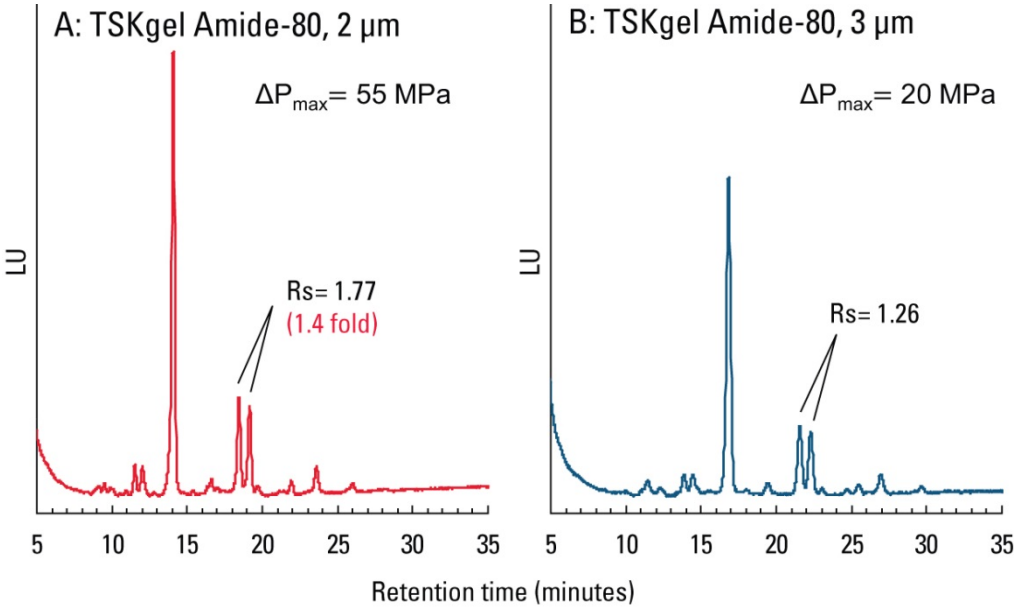
- Separation of the four nucleobases on the TSKgel SuperSW mAb HTP column using conventional SEC conditions are shown above.
- As expected, due to the similarities in molecular masses between the four compounds, significant interference is observed amongst the peaks of interest, particularly the three pyrimidine derivatives, when separated on the TSKgel SuperSW mAb HTP column under SEC conditions.
- The late elution of adenine (relative to the other 3 compounds) may be attributed to possible interactions between the stationary phase and the derivatized purine compound, leading to a shift towards longer retention time.

Separation of Four Nucleobases using TSKgel SuperSW mAb HTP Column in HILIC Mode at pH 7.4



- The separation of 4 nucleobases using the TSKgel SuperSW mAb HTP column in HILIC mode with 15 mmol/L ammonium bicarbonate, pH 7.4 is shown above.
- It is important to note that the order of elution of the analytes does not correlate with their molecular mass (as in SEC separations), but instead is based on their relative hydrophilicity.

Separation of PA-glycans from Mouse Monoclonal Antibody using TSKgel Amide-80, 2 µm HILIC Column

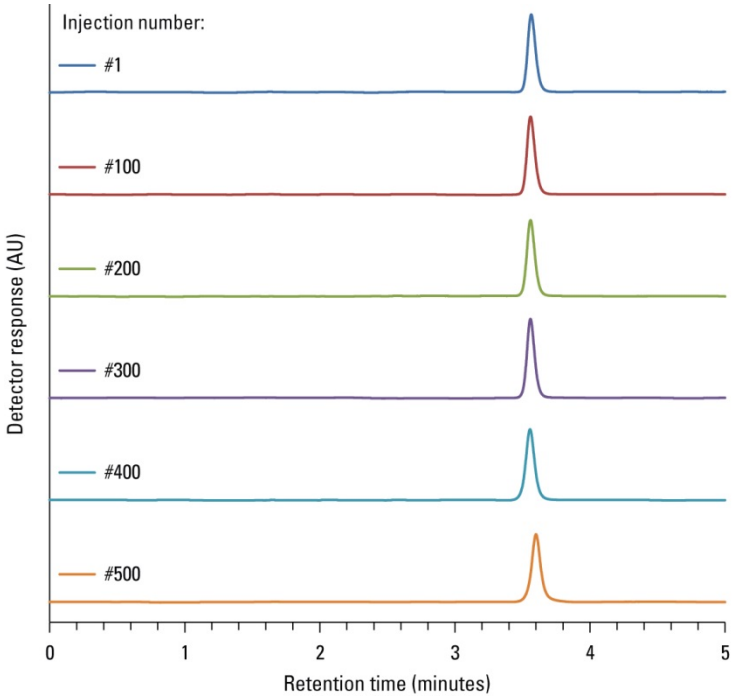


Columns:	A: TSKgel Amide-80, 2 µm, 2.0 mm ID × 15 cm B: TSKgel Amide-80, 3 µm, 2.0 mm ID × 15 cm
Mobile phase:	A: 200 mmol/L acetic acid+ triethylamine (pH 7.3) B: acetonitrile
Gradient:	75%B (0 - 5min), 75 - 50%B (5 - 80min, linear)
Flow rate:	0.5 mL/min
Temperature:	40 ˚C
Detection:	fluorescence λex: 315 nm, λem: 380 nm
Injection vol.:	50 µL
Sample:	pyridylaminated oligosaccharides released from mAb-1 (mouse)

- A comparison was done on the separation of pyridylaminated oligosaccharides released from mouse IgG on TSKgel Amide-80, 2.0 mm ID × 15 cm columns differing in particle size.
- As seen in the figure above, the TSKgel Amide-80, 2 µm showed a 1.4-fold higher resolution of PA-glycan peaks compared to the TSKgel Amide-80, 3 µm column.
- With similar selectivity, method transfer from a 3 µm to a 2 µm column is easily accomplished.
- Note that the TSKgel Amide-80, 2 µm column performed at a pressure of approximately 55 MPa during the gradient elution when run at the flow rate of 0.5 mL/min.
- A UHPLC system or a conventional HPLC system with a maximum pressure rating of greater than 40 MPa is required if the separation is run at this higher flow rate.

Ref: Tosoh Application note AN83

Stability of TSKgel Amide-80, 2 µm Column



Column:	TSKgel Amide-80, 2µm, 3.0 mm ID x 15 cm + TSKgel Amide-80 guard column, 2 µm, 2.0 mm ID × 1 cm
Mobile phase:	H_2O / acetonitrile = 15/85 (v/v)
Flow rate:	0.43 mL/min
Detection:	UV @ 254 nm
Temperature:	40 °C
Injection vol.:	2 µL
Sample:	uracil

- The figure above demonstrates the highly reproducible performance of a TSKgel Amide-80, 2 µm column after 500 injections of uracil.
- This figure also indicates the uncompromised packing integrity of the stationary phase over repeated consecutive injections.
- Throughout the study, the analytical column was protected by a 2 mm ID × 1 cm TSKgel Amide-80 guard column, which was packed with the same support as that contained in the analytical column.
- The usage of a guard column is recommended, as the lifetime of the column will be enhanced.

Conclusions

- The 2 µm TSKgel Amide-80 column is useful for the separation of a number of polar analytes.
- The 2 µm TSKgel Amide-80 column yielded better sensitivity, efficiency and resolution with similar selectivity to a 3 µm Amide-80 column – this is useful for easy method transfer.
- Ultra-high fast separation with TSKgel Amide-80, 2 µm column for a set of hydrophilic molecules within a minute is a great advantage.
- The 2 µm column could separate polar compounds including a water-soluble vitamin, nucleobases and glycans.
- Separation of four nucleobases using a TSKgel SuperSW mAb HTP column in HILIC mode at pH 7.4 shows that this UHPLC compatible column can also be used under HILIC mode, exploiting the hydrophilic diol coating chemistry.
- The 2 µm TSKgel Amide-80 column is very stable. Since the particle size is small, there is a greater risk of losing column efficiency, so it is important that the column is properly maintained.
- The use of a guard column, filtering samples, intermittent cleaning of the column, etc. are highly recommended to extend the column lifetime.
- Overall the HILIC mode of chromatography is very useful for the separation of polar analytes.