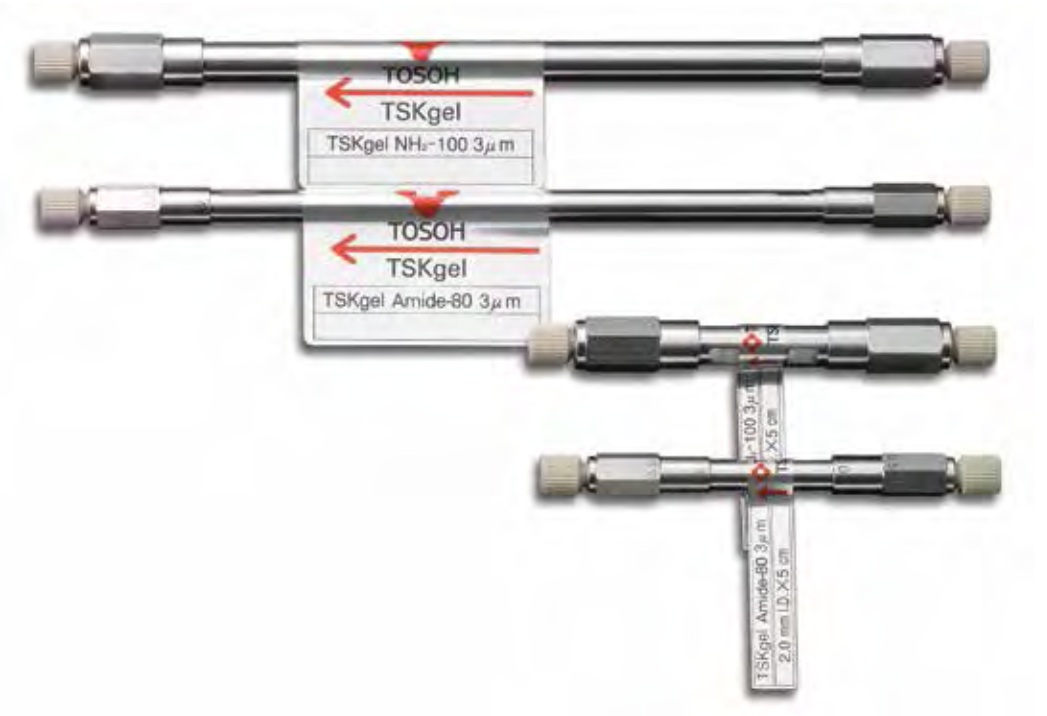
TSKgel Amide-80

TSKgel NH_2-100

HILIC Tips:

- TSKgel HILIC columns are offered in stainless steel. Stainless steel (SS) frits are embedded in the body of the column end-fittings of the metal columns. The nominal frit size for SS columns is engraved in the end-fittings.
- Halide salts corrode stainless steel tubing, fitting, and frits. Do not store SS columns in a mobile phase containing NaCl and, where possible, use another salt in the operating buffer.
- Good laboratory procedures demand that the analytical column be protected by a guard column. Guard cartridges and packed guard columns are available for use with TSKgel HILIC columns.
- Column shipping solvents are: 85% acetonitrile - 15% water for TSKgel Amide-80, 3 µm & HR columns and TSKgel NH_2-100 columns; 75% acetonitrile - 25% water for 5 µm & 10 µm TSKgel Amide-80 columns.
- TSKgel HILIC columns are supplied with an Inspection Data Sheet, which includes a QC chromatogram and test data, and an OCS Sheet summarizing the recommended operating conditions for optimum column performance.
- A separate TSKgel Column Instruction Manual that reviews general guidelines for column installation and care, as well as troubleshooting tips for commonly encountered problems, can be downloaded from the Tosoh Bioscience LLC website (www.tosohbioscience.com).



About

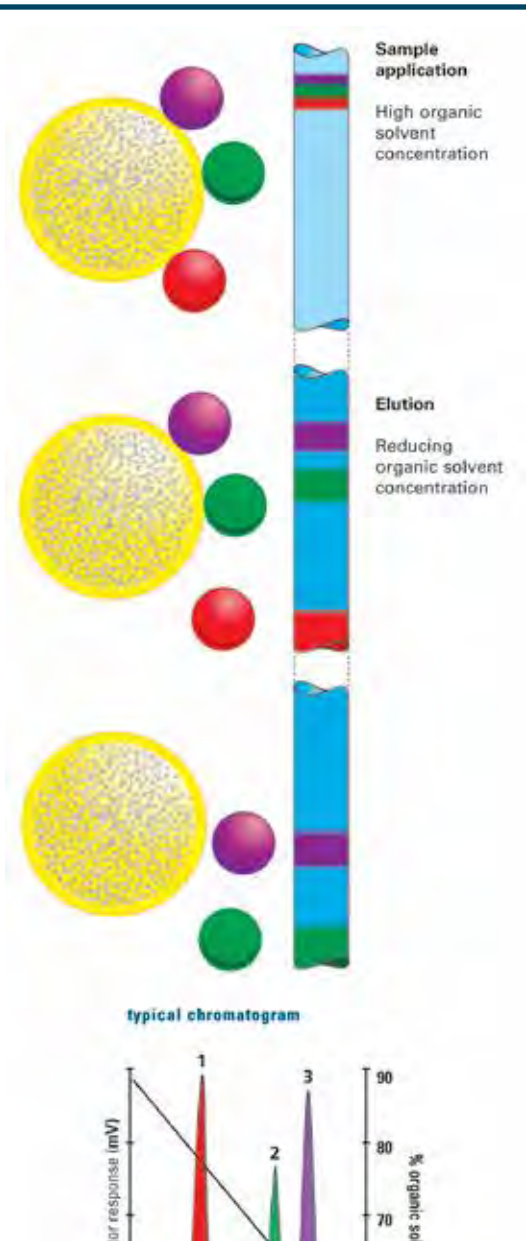
Normal Phase Chromatography (NPC) uses a polar stationary phase and a non-polar mobile phase. The polar surface can either be obtained by derivatizing the support with a ligand containing a polar functional group, or it can be the original (polar) support, as in the case of silica gel.

The popularity of bare silica gel declined after the introduction of bonded phases, since bonded phases feature shorter equilibration times and allow the use of gradient elution. Cyano, amino, and diol bonded phases are the most common normal phase column types.

The order of elution in normal phase is opposite that found in reversed phase chromatography for the same mixture of compounds. 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.

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 mechanism using a mobile phase mixture of acetonitrile and ammonium acetate buffer. In contrast to the retention behavior in reversed phase, in HILIC, solutes will be retained longer when increasing the percent acetonitrile.

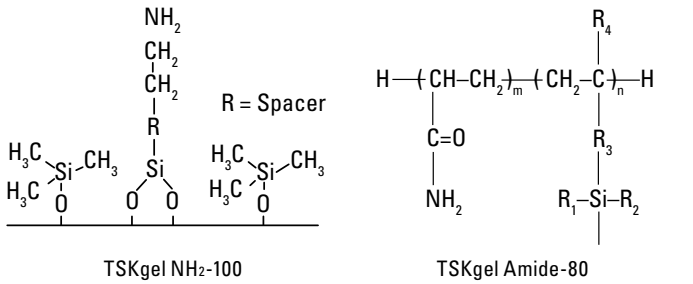
Figure 1: Hydrophilic Interaction Chromatography



TSKgel HILIC Chromatography Columns

The TSKgel HILIC chromatography column line consists of TSKgel Amide-80 and TSKgel NH_2-100. TSKgel Amide-80 columns are covalently bonded with carbamoyl groups. Offering a different selectivity from the TSKgel Amide-80 columns, the TSKgel NH_2-100 amino-bonded phase columns offer superior chemical stability. The structures of the TSKgel Amide-80 and NH_2-100 resins are shown in Figure 2. See Table 1 for details on these two TSKgel HILIC columns.

Figure 2: Structures of TSKgel HILIC resins



TSKgel NH_2-100 TSKgel Amide-80

*The spacer (R) contains secondary as well as tertiary amino groups.

Table 1: Features of TSKgel HILIC columns

	TSKgel Amide-80	TSKgel NH_2-100
Bonded Phase	Does not react with reducing sugars. Anomer formation can be prevented by raising mobile phase temperature up to 80 °C.	More stable than conventional amino phases due to a special endcapping prior to introduction of aminoalkyl groups. Amino-bonded phase can react with a reducing sugar to form a Schiff base
Detectors	Can be used with evaporative light scattering (ELS) and mass spec (MS) detectors	
Mobile Phase	Stable in 100% organic eluents	
Solutes	Retains very polar drug candidates and drug metabolites	
Applications	Saccharides	
	Polyols (polyalcohols)	
	Drug metabolites	Methotrexate polyglutamate derivatives
	Peptides	Pyridylaminated oligosaccharides
	Melamine and cyanuric acid	Water-soluble vitamins
	Oligonucleotides	Nucleic acid fragments
	Polar compounds in fermentation broth	
	Cytosine	

About: TSKgel Amide-80 HILIC Chromatography Columns

TSKgel Amide-80 columns are packed with 3, 5, or 10 µm spherical silica particles that are covalently bonded with carbamoyl groups. The amide stationary phase provides a unique selectivity under regular normal phase conditions or in the hydrophilic interaction mode of chromatography. TSKgel Amide-80 columns possess superior stability in aqueous/organic solvent systems, an advantage over other hydrophilic stationary phases such as traditional amino-bonded phase columns. In addition, it is ideally suited for sensitive LC/MS analysis of water-soluble polar compounds because the high organic content in the mobile phase provides rapid evaporation of solvent during electrospray ionization.

Also available within this line is a TSKgel Amide-80 HR column in 5 µm, 4.6 mm ID × 25 cm size. These columns are prepared from the same 5 µm packing material and thus offer the same selectivity as existing 5 µm TSKgel Amide-80 columns. In contrast to traditional 4.6 mm ID × 25 cm TSKgel Amide-80 columns, the new TSKgel Amide-80 HR columns offer higher specifications for plate count (N = >18,000 vs. 8,000). Because HR columns are prepared from the same bonded phase lots as standard 5 µm TSKgel Amide-80 columns, the only change you will notice is more efficient chromatography!

Attributes and Applications

Table 2 lists the attributes of TSKgel Amide-80 columns. Target applications for the TSKgel Amide-80 columns include the analysis of saccharides, glycans, oligosaccharides, peptides, and polar compounds from natural products. Unique advantages of the TSKgel Amide-80 phase for saccharide analysis include a novel hydrogen bonding retention mechanism between hydroxyl groups of the sample and the carbamoyl group in the stationary phase. The stationary phase does not react with reducing sugars and can be used at elevated temperatures (4 to 80 °C) to prevent peak splitting of carbohydrates that can occur at lower temperatures.

Table 2: Product attributes

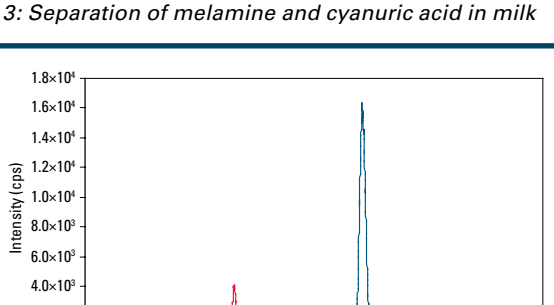
Attribute	Value
Pore size (silica)*	8 nm
Particle size	3 µm, 5 µm, or 10 µm
pH stability	2.0-7.5
Functional group	carbamoyl
Max. temperature	80 °C
Surface area (m^2/g)	450

*The pore size of the bonded phase is indicated by the number in the product description, in this case TSKgel Amide-80 has 8 nm nominal pore size. The nominal pore size of the starting base silica is 10 nm.

Melamine and Cyanuric Acid in Milk

To aid chemists charged with the determination of melamine and related products in milk, Tosoh scientists developed a method for the simultaneous determination of melamine and cyanuric acid by HILIC/MS/MS using a 3 µm TSKgel Amide-80 column. Milk was spiked with melamine and cyanuric acid standards to serve as a model sample. High recovery and excellent resolution was obtained for both compounds, as shown in Figure 3.

Figure 3: Separation of melamine and cyanuric acid in milk

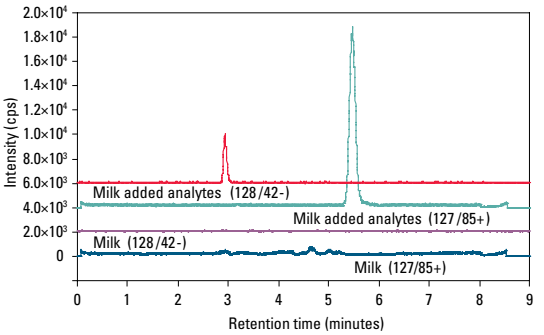


Column:	**TSKgel Amide-80, 3 µm, 2.0 mm ID × 15 cm**
Mobile phase:	A: 0.05% formic acid in H_2O
	B: 0.05% formic acid in ACN
	A/B = 25/75
Flow rate:	0.2 mL/min
Temperature:	40 °C
Injection vol.:	5 µL
Instrument:	Q TRAP® (AB Sciex)
Ion source:	ESI
	127/85+ (melamine)
	128/42- (cyanuric acid)

Melamine and Cyanuric Acid in Milk, Continued

Multiple Reaction Monitoring is a mode of MS/MS that yields maximum sensitivity and selectivity for known target analytes. Figure 4 shows the results of this type of mass analysis on unspiked and spiked milk samples. The figure demonstrates that the original milk sample did not contain any amount of either melamine or cyanuric acid. After adding the compounds to the milk sample, melamine and cyanuric acid were independently detected, with more than sufficient resolution between the compounds.

Figure 4: Multiple Reaction Monitoring (MRM) chromatograms of milk and spiked milk samples - 10 ppb each



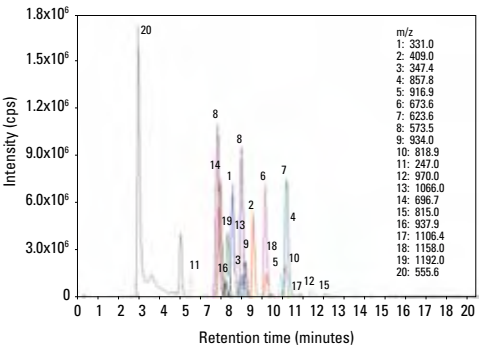
Column:	**TSKgel Amide-80, 3 µm, 2.0 mm ID × 15 cm**
Mobile phase:	A: 0.05% formic acid in H_2O
	B: 0.05% formic acid in ACN
	A/B = 25/75
Flow rate:	0.2 mL/min
Temperature:	40 °C
Injection vol.:	5 µL
Instrument:	Q TRAP (AB Sciex)
Ion source:	ESI
	127/85+ (melamine)
	128/42- (cyanuric acid)

Tryptic Digest

Scientists have begun to employ the hydrophilic stationary phase in separations of natural product extracts, peptide digests, and other polar compound mixtures. Often, these complex mixtures are analyzed using the power of mass spectrometry in conjunction with liquid chromatography. TSKgel Amide-80 columns are advantageous for these applications because unwanted secondary ionic interactions from residual silanols can be eliminated by adding trifluoroacetic acid (TFA) to the mobile phase. The use of "mass-spec friendly" TFA eliminates extra steps involved with removing salts or non-volatile acids required by amino-bonded columns to eliminate ionic interactions.

Figure 5 details the use of a 5 µm TSKgel Amide-80 column for the separation of a β-lactoglobulin tryptic digest, with separation achieved within 12 minutes.

Figure 5: Separation of β-lactoglobulin tryptic digest

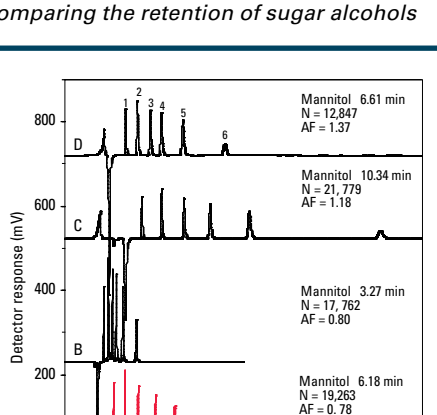


Column:	**TSKgel Amide-80, 5 µm, 2.0 mm ID × 15 cm**
Mobile phase:	A: 0.1% TFA in H_2O
	B: 0.1% TFA in ACN
Gradient:	0 min (95%B) 15 min (50%B) 17 min (50%B)
Flow rate:	0.2 mL/min
Injection vol.:	1 µL
Sample:	β-lactoglobulin tryptic digest
Instrument:	Q TRAP (AB Sciex)
Ion Source:	ESI+

Sugar Alcohols

Figure 6 shows comparative chromatograms of the separation of sugar alcohols using a TSKgel Amide-80 column and competitive HILIC columns. The TSKgel Amide-80, 3 µm column showed high column efficiency for mannitol, while the selectivity for saccharides was as good or better for the TSKgel Amide-80 column compared to the competitive HILIC columns.

Figure 6: Comparing the retention of sugar alcohols

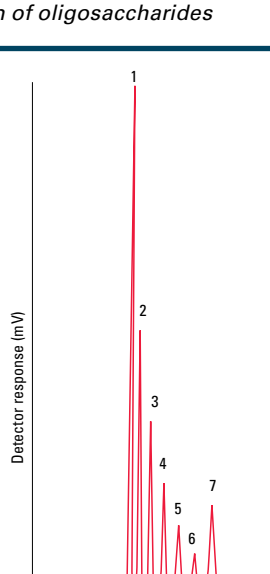


Columns:	**A: TSKgel Amide-80, 3 µm, 4.6 mm ID × 15 cm**
	B: Luna® NH_2, 4.6 mm ID × 15 cm
	C: Polyamine®, 4.6 mm ID × 25 cm
	D: NH_2P-50 4E®, 4.6 mm ID × 25 cm
Mobile phase:	H_2O/ACN (25/75)
Flow rate:	1.0 mL/min
Detection:	RI
Temperature:	40 °C
Injection vol.:	10 µL
Samples:	1. ethylene glycol
	2. glycerin
	3. erythritol
	4. xylitol
	5. mannitol
	6. inositol

Carbohydrates

A TSKgel Amide-80 column can separate oligosaccharides very rapidly and efficiently. Figure 7 shows a separation of a β-cyclodextrin hydrolysate in less than 10 minutes. The peak numbers indicate the degree of polymerization of the repeating base sugar in the oligosaccharide with 1 representing a single glucose unit, 2 a dimer, etc.

Figure 7: Separation of oligosaccharides

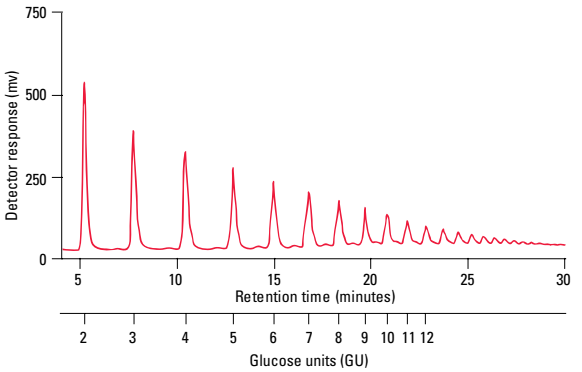


Column:	**TSKgel Amide-80, 5 µm, 4.6 mm ID × 25 cm**
Mobile phase:	ACN/H_2O (55/45)
Flow Rate:	1.0 mL/min
Detection:	RI
Temperature:	25 °C
Sample:	2 µL, β-cyclodextrin hydrolysate, 1-7 indicate degree of polymerization (4.6 g/L)

Carbohydrates

TSKgel Amide-80 chemistry is ideally suited for the separation of carbohydrate structures. Figure 8 shows the high resolution separation of a 2-aminobenzamide (2AB) labeled dextran ladder within 30 minutes on a TSKgel Amide-80, 3 µm column. This ladder can be used as a calibration standard for HPLC and MS analysis of glycans. The ladder contains glucose homopolymer species from degree of polymerization (dp) 1 to dp 22 (i.e. the glucose monomer GU1-2AB to GU22-2AB).

Figure 8: 2-AB Labeled Glucose Homopolymer (GHP) Ladder



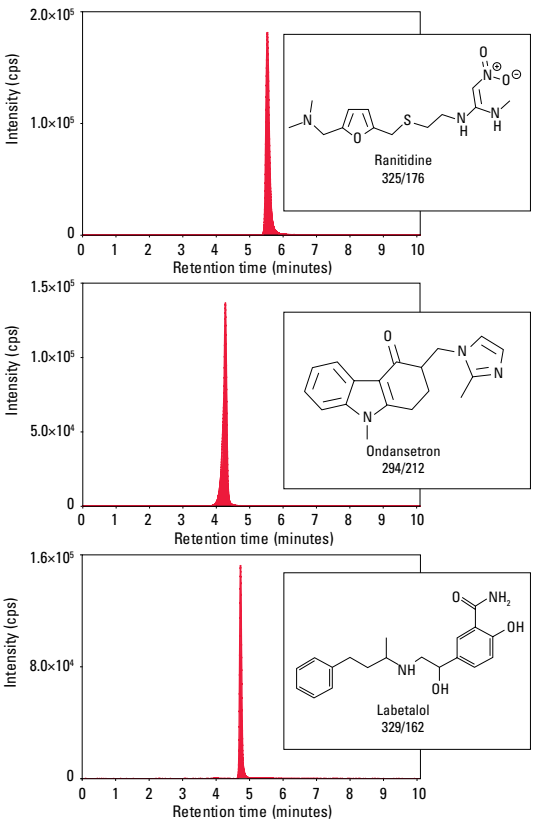
Column:	**TSKgel Amide-80, 3 µm, 2.0 mm ID × 15 cm**
Mobile phase:	A: 50 mmol/L ammonium formate, pH 4.3
	B: ACN
Gradient:	0-35 min - 75-35%B
Flow rate:	0.22 mL/min
Detection:	fluorescence
	Ex: 360 nm, Em: 425 nm
Temperature:	50 °C
Injection vol.:	3 µL
Sample:	CAB-GHP dextran ladder
	(Ludger: ~ 300 fmol for GU2)

*Courtesy of K. Darsow & H. Lange, Institute of Bioprocessing, University of Nurnberg/ Erlangen

Polar Drugs

TSKgel Amide-80 columns are also a valuable tool for the analysis of small molar mass polar drugs that are not sufficiently retained on reversed phase columns. Figure 9 shows the separation of polar drug standards in HILIC mode using a 3 µm TSKgel Amide-80 column coupled with electrospray ionization mass spectroscopy (ESI/MS). Due to the high organic content of the eluent, HILIC analysis provides increased detection sensitivity.

Figure 9: Separation of polar drug standards

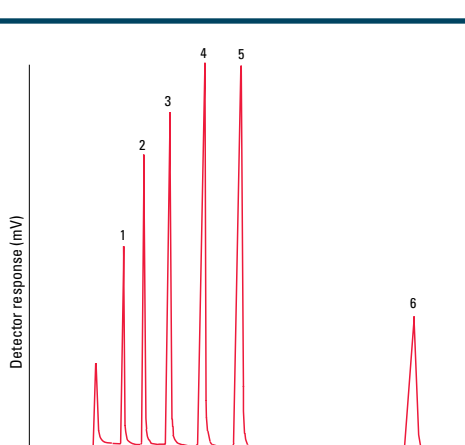


Column:	**TSKgel Amide-80, 3 µm, 2.0 mm ID × 15 cm**
Mobile phase :	A: 10 mol/L ammonium formate, pH 3.75
	B: ACN
Gradient :	0 min (90%B) 10 min (40%B) 13 min (40%B)
Flow rate :	0.2 mL/min
Injection vol.:	5 µL (50 µg/L)
Samples:	ranitidine
	ondansetron
	labetalol
Instrument :	Q TRAP (AB Sciex) LC/MS/MS
Ion Source:	ESI+

Polyalcohols

Polyalcohols are typically separated using a TSKgel Amide-80 column with a mobile phase of organic solvent and water as shown in Figure 10.

Figure 10: Separation of polyalcohols



Column:	**TSKgel Amide-80, 5 µm, 4.6 mm ID × 25 cm**
Mobile phase:	ACN/H_2O (75/25)
Flow rate:	1.0 mL/min
Detection:	RI
Temperature:	25 °C
Samples:	1. ethylene glycol, 10 mmol/L, 20 µL 2. glycerin, 10 mmol/L, 20 µL 3. erythritol, 10 mmol/L, 20 µL 4. xylitol, 10 mmol/L, 20 µL 5. mannitol, 10 mmol/L, 20 µL 6. inositol, 4 mmol/L, 20 µL

About: TSKgel NH_2-100 HILIC Chromatography Columns

TSKgel NH_2-100 amino columns expand the range of TSKgel columns for hydrophilic interaction chromatography (HILIC). Offering a different selectivity from the well known TSKgel Amide-80 columns, these novel amino-bonded phase columns stand out by providing much improved chemical stability, a prerequisite for achieving reproducible and reliable results.

TSKgel NH_2-100 columns are packed with 3 µm silica particles containing 10 nm pores. A novel bonding strategy was adopted to improve chemical stability of the bonded phase. First, the silica is reacted with a trimethylsilane endcapping reagent at a low stoichiometric ratio before reacting residual and accessible silanol groups with trifunctional alkylaminosilane reagent. The resulting bonded phase provides a better safeguard against hydrolysis of the underlying silica.

TSKgel NH_2-100 columns are unique in that the bonded phase ligand not only has a terminal primary amino group as expected, but that the spacer also incorporates secondary as well as tertiary amino groups. Anionic compounds are retained on the column by ionic interaction. This allows for the use of gradients in salt concentration in addition to gradient elutions with acetonitrile. Since the TSKgel NH_2-100 columns have cationic sites, the columns can be used as mixed mode columns under some conditions.

Also available within this line is a TSKgel NH_2-100 DC column that connects directly to TSKgel reversed phase columns. The DC in TSKgel NH_2-100 DC emphasizes this Direct Connect aspect. This column has the same packing material as the TSKgel NH_2-100 columns, so therefore shows high retention for hydrophilic compounds/ions. A male-type outlet end-fitting enables the direct connection to the screw-type end-fitting of a TSKgel reversed phase column. This allows for the simultaneous separation using a linear gradient of an active pharmaceutical ingredient (API) and its counterion without the loss of column efficiency normally experienced when connecting two columns with capillary tubing.

Attributes and Applications

Product attributes of the TSKgel NH_2-100 columns are listed in Table 3. TSKgel NH_2-100 columns are well suited for the analysis of all types of hydrophilic compounds, including carbohydrates and peptides. Due to a high ligand density and large surface area, these columns show stronger retention of polar compounds than TSKgel Amide-80 columns.

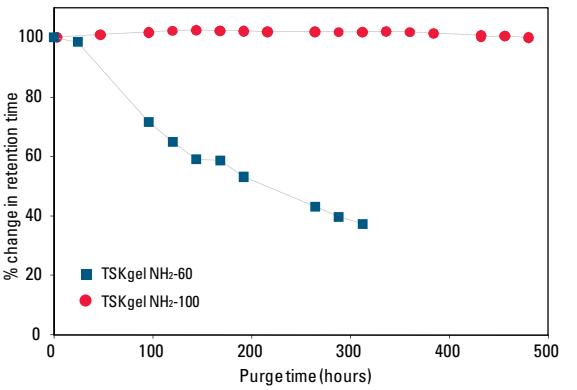
Table 3: TSKgel NH_2-100 product attributes

Attribute	Value
Pore size (silica)	10 nm
Particle size (mean)	3 µm
pH stability	2.0-7.5
Functional group	alkylamine
Temperature range (°C)	10-50
Surface area (m^2/g)	450

Performance Data

Figure 11 shows the result of a long term chemical stability comparison between the more recently developed TSKgel NH_2-100 column and the older TSKgel NH_2-60 column. Both columns were purged for 300 hours in 25% H_2O/75% ACN and you can see that 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 TSKgel NH_2-100 HILIC column, the retention time of inositol decreased less than 20%.

Figure 11: Chemical stability study

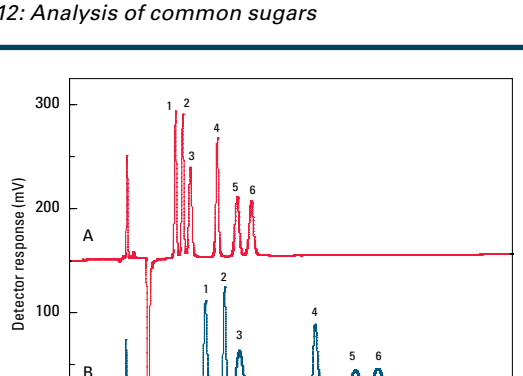


Columns:	**TSKgel NH_2-100, 3 µm, 4.6 mm ID × 15 cm** **TSKgel NH_2-60, 5 µm, 4.6 mm ID × 25 cm**
Mobile phase:	H_2O/ACN (25/75)
Flow Rate:	1.0 mL/min
Detection:	RI
Temperature:	40 °C
Injection vol.:	10 µL
Sample:	inositol

Sugars

A TSKgel NH_2-100 column was used to analyze sugars - a typical analysis done in the food industry. Two different eluents were used to compare retention times and peak shapes. As shown in Figure 12, a mixture of water and acetone provided superior resolution within 6 minutes; whereas an eluent mixture of triethylaluminium-hydroxymethylfuranal and acetone did not produce as sharp of peaks and the retention time was over 4 minutes longer.

Figure 12: Analysis of common sugars

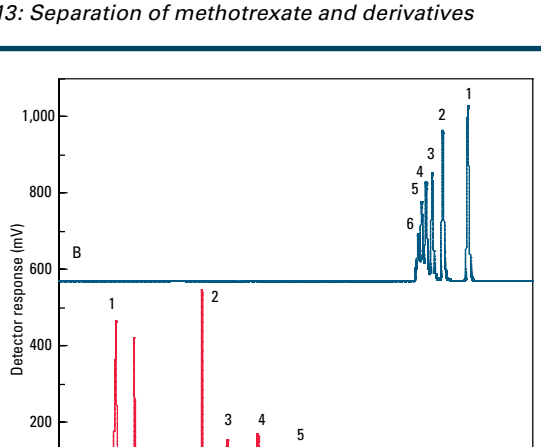


Column:	**TSKgel NH_2-100, 3 µm, 4.6 mm ID × 15 cm**
Mobile phase:	A: H_2O/acetone (25/75)
	B: 100 mmol/L TEA-FA, pH 10.0/acetone (25/75)
Flow rate:	1.0 mL/min
Detection:	RI
Temperature:	50 °C
Injection vol.:	10 µL
Samples:	1. fructose
	2. sorbitol
	3. glucose
	4. sucrose
	5. maltose
	6. lactose

Methotrexate and Derivatives

Figure 13 shows the results of methotrexate and its derivatives ($MTXPG_{2-7}$) analyzed on TSKgel NH_2-100, 3 µm HILIC and TSKgel ODS-100V, 3 µm reversed phase narrow bore columns. The MTX and polyglutamate derivatives were eluted in the order of the number of glutamate groups in their molecules on the TSKgel NH_2-100 HILIC column, but eluted in reverse order on the TSKgel ODS-100V column. Despite the early elution of MTX and $MTXPG_2$ on the TSKgel NH_2-100 HILIC column, the overall separation is better than what can be accomplished on the C18 column.

Figure 13: Separation of methotrexate and derivatives

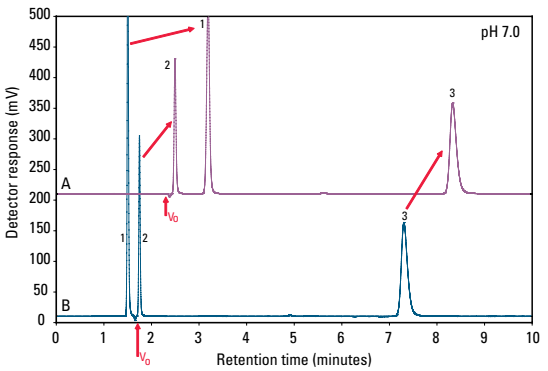


Columns:	**A. TSKgel NH_2-100, 3 µm, 2.0 mm ID × 15 cm**
	B. TSKgel ODS-100V, 3 µm, 2.0 mm ID × 15 cm
Mobile phase:	A: A) H_2O/ACN (10/90) + 0.1% TFA
	B) H_2O + 0.1% TFA
	B: A) H_2O/ACN (10/90) + 0.1% TFA
	B) ACN + 0.1% TFA
Gradient:	0 min (0%B) 15 min (40%B) 17 min (0%B)
Flow rate:	0.20 mL/min
Detection: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

Drug and Counter Ions at pH 7.0

Maleic acid and p-toluene sulfonic acid are commonly used as counter ions in pharmaceutical preparations. Both of these organic acids are hydrophilic and are not retained on a TSKgel ODS-100V reversed phase column at pH 7.0 in 70% methanol eluent (Figure 14). With the connection of a TSKgel NH_2-100 DC column prior to the TSKgel ODS-100V column, the simultaneous determination of maleic acid and the API desipramine becomes possible. Maleic acid is slightly retained on the TSKgel NH_2-100 DC column by an anion exchange interaction. Desipramine, on the other hand, does not interact with the protonated amino groups as it is positively charged.

Figure 14: Simultaneous determination of maleic acid and the API desipramine at pH 7.0

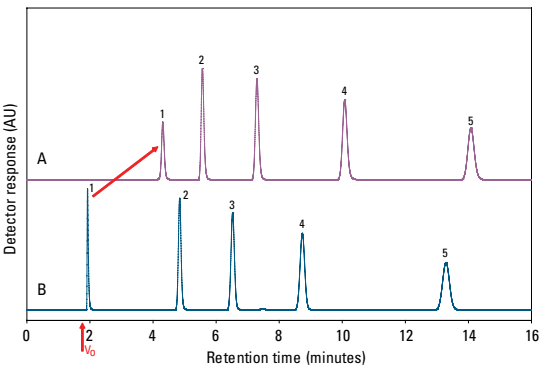


Columns:	**A: TSKgel NH_2-100 DC, 3 µm, 4.6 mm ID × 5 cm + TSKgel ODS-100V, 3 µm, 4.6 mm ID × 15 cm**
	B: TSKgel ODS-100V, 3 µm, 4.6 mm ID × 15 cm
Mobile phase:	50 mmol/L phosphate buffer, pH 7.0/MeOH = 30/70
Flow rate:	1.0 mL/min
Detection:	UV @ 210 nm
Temperature:	40 °C
Inj. volume:	5 µL
Samples:	1.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

Cold Medicine Ingredients

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, 3 µm column. Direct Connection (DC) of the TSKgel NH_2-100 DC, 3 µm column to a TSKgel ODS-100V, 3 µm column allows for the simultaneous determination of APIs and guaiacol sulfonic acid in a single run as shown in Figure 15.

Figure 15: Separation of cold medicine ingredients



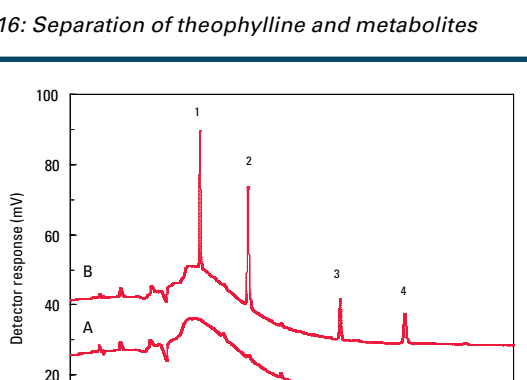
Columns:	**A) TSKgel NH_2-100 DC, 3 µm, 4.6 mm ID × 5 cm + TSKgel ODS-100V, 3 µm, 4.6 mm ID × 15 cm**
	B) TSKgel ODS-100V, 3 µm, 4.6 mm ID × 15 cm
Mobile phase:	50 mmol/L NaH_2PO_4, pH 2.5/MeOH = 65/35
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	40 °C
Injection vol.:	5 µL
Samples:	1. guaiacol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

Therapeutic Drug Monitoring

Therapeutic drug monitoring (TDM) by HPLC is required for several drug classes, as the effective dosage range, varying between insufficient activity and toxic levels, is patient dependent. Although a reversed phase column is typically adopted for separating drugs in blood samples, hydrophilic compounds show poor retention times on an ODS column. A TSKgel NH_2-100, 3 µm HILIC column was investigated for the separation of hydrophilic drugs and its metabolites in blood.

Theophylline is a medication that is used for treating airway spasms in people with asthma or chronic obstructive pulmonary disease (COPD). Serum levels of this drug correlate well with both therapeutic and toxic effects. As demonstrated in Figure 16, spiked samples of theophylline and its metabolites could be separated successfully with a TSKgel NH_2-100 column using an off-line deproteinization procedure.

Figure 16: Separation of theophylline and metabolites



Column: **TSKgel NH_2-100, 3 µm, 2.0 mm ID × 15 cm**

A: Supernatant of serum deproteinated with 10-fold ACN
B: Supernatant of spiked serum deproteinated with 10-fold ACN

Mobile phase:	A: 0.1 mol/L triethylamine-formic acid, pH 10.0/ACN (5/95) B: 0.1 mol/L triethylamine-formic acid, pH 10.0/ACN (50/50)
Gradient:	0 min-2 min (0%B) 2-30 min (80%B) 30-32 min (0%B)
Flow rate:	0.25 mL/min
Detection:	UV @ 254 nm
Temperature:	40 °C
Injection vol.:	10 µL
Samples:	1. theophylline 2. 3-methylxanthine 3. 1,3-dimethyluric acid 4. 1-methyluric acid (concentration: 10 mg/L for each)