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Application Note

Alternate Selectivity for Polar Compounds in Hydrophilic Interaction Liquid Chromatography (HILIC) Using a New Amino Type HILIC Column

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Hydrophilic interaction liquid chromatography (HILIC) offers unique advantages for the separation of very polar compounds when compared to reversed-phase chromatography. A new silica based HILIC phase was developed to provide additional selectivity options in HILIC separations. The separation of water soluble vitamins on the new TSKgel NH2-100 HILIC column and on the well known TSKgel Amide-80 HILIC column demonstrates the differences in selectivity.

INTRODUCTION

HILIC is used primarily to separate polar and hydrophilic compounds. Target applications for HILIC include the analysis of saccharides, glycosides, oligosaccharides, peptides and hydrophilic drugs. Altering selectivity plays a major role in maximizing resolution.

HYDROPHILIC INTERACTION CHROMATOGRAPHY

HILIC has similarities to normal phase chromatography with regard to the nature of the stationary phase. However, the eluents used for HILIC are similar to those known from reversed phase chromatography, e.g mixtures of acetonitrile and water or aqueous buffers, applied in isocratic or gradient mode. Hydrogen bonding and dipole–dipole interactions are the dominating retention mechanisms in HILIC mode. The number of polar groups, as well as the conformation and solubility of the sample in the mobile phase determine the elution order.

HILIC PHASES

Typical HILIC stationary phases are silica or polymer particles carrying polar functional groups (e.g., amino, amide or zwitterionic groups). Conventional amino type HILIC columns have limited stability in aqueous solutions but sometimes the selectivity of an amino ligand better suits the target application. Therefore, we developed a new HILIC phase, which combines the amino ligand functionality with high durability.

The new amino type HILIC phase is based on a 3 µm silica particle with 100 Å pores, which is treated with a special endcapping procedure. Amino groups are introduced step wisely after endcapping (Figure 1).

SCHEMATIC DIAGRAM OF THE NEW HILIC PHASE

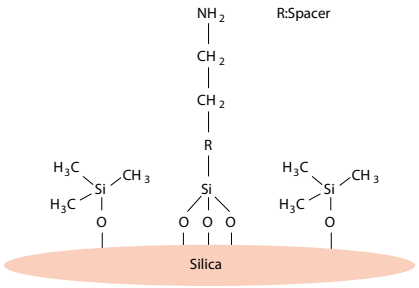

Figure 2

The amino groups act as HILIC functional groups. Because of a high ligand density and large surface area TSKgel NH2-100 3 µm columns show the strongest retention for polar compounds among the commercially available HILIC columns.

H-U-PLOTS OF TSK-GEL AMIDE-80 AND NH-2-100 HILIC

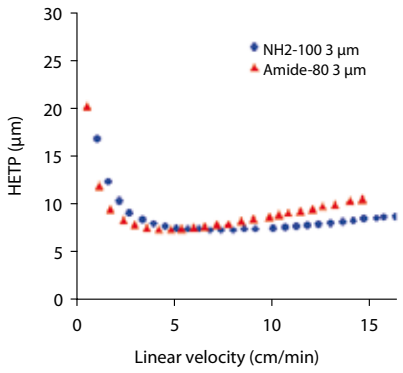

Figure 2

Conditions:

Columns:	TSKgel NH2-100 3 µm and TSKgel Amide-80 3 µm (4.6 mm ID x 15 cm L each)
Sample:	Uracil
Eluent:	H2O/acetonitrile = 10/90
Flow rates:	0.1 2.4 mL/min
Temp.:	40 °C
Detection:	UV @ 254 nm

Figure 2 shows the H-u plots of the TSKgel Amide-80 and NH2-100 HILIC phases. The optimum HETP for the amino type column is reached at a flow-rate of 1.2 mL/min at a pressure of 6 MPa. At increased flow-rates the H-u-curve is relatively flat. This allows using this column for fast separations at elevated flow rates without impairing separation efficiency.

The availability of two TSK-GEL HILIC phases offering different selectivity features allows for adopting both, the stationary as well as the mobile phase to optimize the separation of a given sample. Available in a particle size of 3 µm, both columns are ideally suited for high efficiency HPLC as well as HPLC–MS analysis

SEPARATION OF WATER SOLBLE VITAMINS

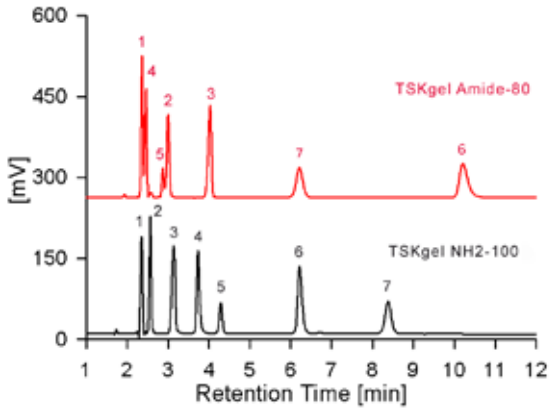

Figure 3

Columns: TSKgel Amide-80 and TSKgel NH2-100
Peaks: 1 = nicotinamide; 2 = vitamin B2;
3 = pyridoxine; 4 = nicotinic acid;
5 = vitamin C; 6 = vitamin B1;
7 = vitamin B12

HILIC ANALYSIS OF WATER SOLUBLE VITAMINS

HILIC separations are performed either in isocratic mode with a high percentage of organic solvent or with gradients starting with high percentage of organic solvent and ending with a high portion of aqueous solvent, which is opposite to reversed phase. The elution order of compounds is usually inversed as well. As a result in HILIC mode polar compounds are very well separated according to increased polarity. We present the analysis of water soluble vitamins (Nicotinamide, Nicotinic acid, Pyridoxine and Vitamins B1, B2, B12 and C) as an example for the separation of polar compounds on the two available TSK-GEL HILIC phases. For difficult separations of the very polar vitamins of the B complex HILIC separation is advantageous over reversed-phase analysis, especially when combined with highly sensitive mass spectrometric detection.

MATERIAL AND METHODS

Columns: TSKgel NH2-100 3 µm, 4.6 mm ID × 15 cm L
TSKgel Amide-80 3 µm, 4.6 mm ID × 15 cm L
Mobile phase: 25 mM phosphate buffer (pH 2.5)/ACN=30/70
Flow rate: 1 mL/min
Temp.: 40 °C
Detection: UV @ 254 nm
Sample: Figure 3: vitamin standard mixture
Figure 4: energy drink (filtrated, diluted in CAN (1:1))
Injection: 5 µL

Figure 3 shows the separation of a standard solution of water soluble vitamins on a TSKgel NH2-100 column compared to the separation on a TSKgel Amide-80 column. Both columns have the same dimension (4.6 mm ID × 15 cm L) and particle size (3 µm). Flow rate and mobile phase were identical as well.
The elution order of the compounds varies when applying the same mobile phase to both columns: The new amino type column shows a stronger retention for nicotinic acid, vitamin C and vitamin B12 while retention of vitamin B1, B2 and pyridoxine is reduced. Figure 4 shows the analysis of a commercially available energy drink on the new amino-type HILIC column, after filtration and addition of the same volume of acetonitrile.

CONCLUSION

TSKgel Amide-80 HILIC columns have been used for years for a broad range of HILIC applications. The new 3 µm TSKgel NH2-100 columns provide an additional selectivity option, when increased retention or alternate selectivity is needed. This new bonded phase provides a powerful tool for robust method development in hydrophilic interaction liquid chromatography.

ANALYSIS OF VITAMINS IN AN ENERGY DRINK

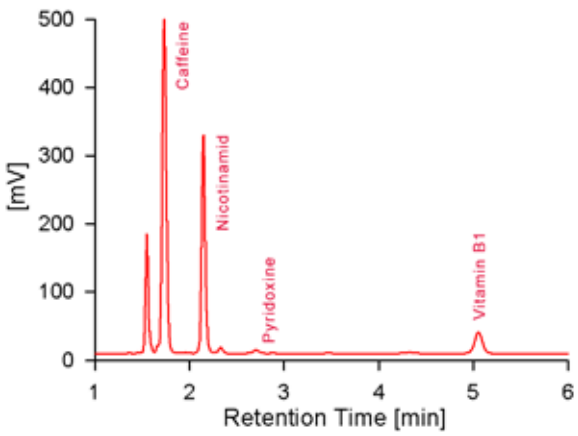

Figure 4

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Application Note

HILIC–MS - High Resolution and Sensitivity for the Analysis of Very Polar Compounds

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Hydrophilic interaction liquid chromatography (HILIC) offers unique advantages for mass spectrometric detection of very polar compounds when compared to reversed phase chromatography. The higher organic content of the eluent in HILIC supports efficient evaporation of the solvent, thus enhancing sensitivity and altering ion suppression. HILIC–MS is particularly suited for LC–MS analysis of glycans or for fast analysis of polar drug substances or metabolites. We present an example for the LC–MS analysis of basic drugs to demonstrate the power of HILIC separations with regard to detection sensitivity and selectivity.

Hydrophilic interaction liquid chromatography (HILIC)[1] is used primarily to separate polar and hydrophilic compounds. Target applications for HILIC include the analysis of saccharides, glycosides, oligosaccharides, peptides and hydrophilic drugs.

HYDROPHILIC INTERACTION CHROMATOGRAPHY

HILIC has similarities to normal phase chromatography (NPC) with regard to the nature of the stationary phase, but the eluents used are similar to those known from reversed phase chromatography (RPC). Typical mobile phases are mixtures of acetonitrile and water or aqueous buffers. Typical stationary phases are silica or polymer particles carrying polar functional groups. TSKgel Amide-80 HILIC columns are packed with spherical silica particles that are covalently bonded with non-ionic carbamoyl groups. They are very well known for the separation of polar compounds such as saccharides[2] or glycans[3].

It is commonly believed that in HILIC the aqueous content of the mobile phase creates a water rich layer on the surface of the stationary phase. This allows for partitioning of solutes between the more organic mobile phase and the aqueous layer. Hydrogen bonding and dipole–dipole interactions are the dominating retention mechanisms in HILIC mode. The number of polar groups, as well as the conformation and solubility of the sample in the mobile phase determines the elution order.

FEATURES OF HILIC–MS

In recent years hyphenated techniques such as GC–MS and LC–MS, are becoming more and more popular. With MS–MS detection techniques a very high sensitivity and selectivity can be reached.

In LC-ESI–MS evaporation of the solvent is crucial for detection sensitivity. A high organic content of the mobile phase is supporting efficient evaporation, thus enhancing MS sensitivity. In RP separation mode the retention of very polar, hydrophilic compounds is usually very low, limiting separation power. This can be improved by using modern RP materials especially designed forseparation of polar compounds. Nevertheless the high aqueous content of the eluent is still impairing LC–MS detection sensitivity.

HILIC separations are performed with gradients starting with high percentage of organic solvent and ending with a high portion of aqueous solvent, which is opposite to RPC.

SELECTIVITY FOR PEPTIDES SEPARATED BY HILIC AND RP CHROMATOGRAPHY

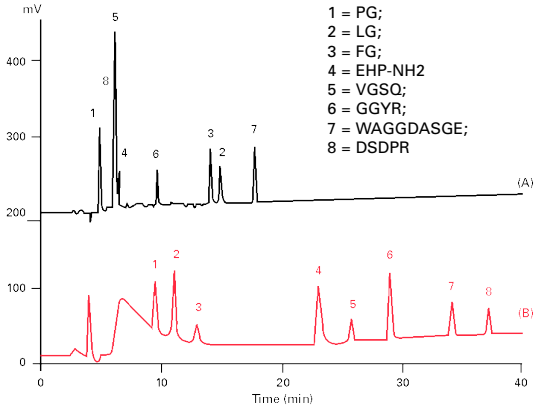

Figure 1

Columns:	(a) TSKgel ODS-80TS 5 µm, 4.6 mm ID x 25 cm L b) TSKgel Amide-80 5 µm, 4.6 mm ID x 25 cm L
Eluents:	A: 0.1% TFA; B: Acetonitrile
Flow rate:	1 mL/min
Gradient:	C18: 5% B to 55% B in 83.3 min HILIC: 97% B to 55% B in 70 min
Sample:	Mixture of small polar peptides
Detection:	UV@215 nm

LC-MS ANALYSIS OF BASIC DRUGS ON HILIC (LEFT) AND RPC (RIGHT) COLUMNS;
RANITIDINE 315/176; ONDANSETRON 294/212; LABETALOL 329/262

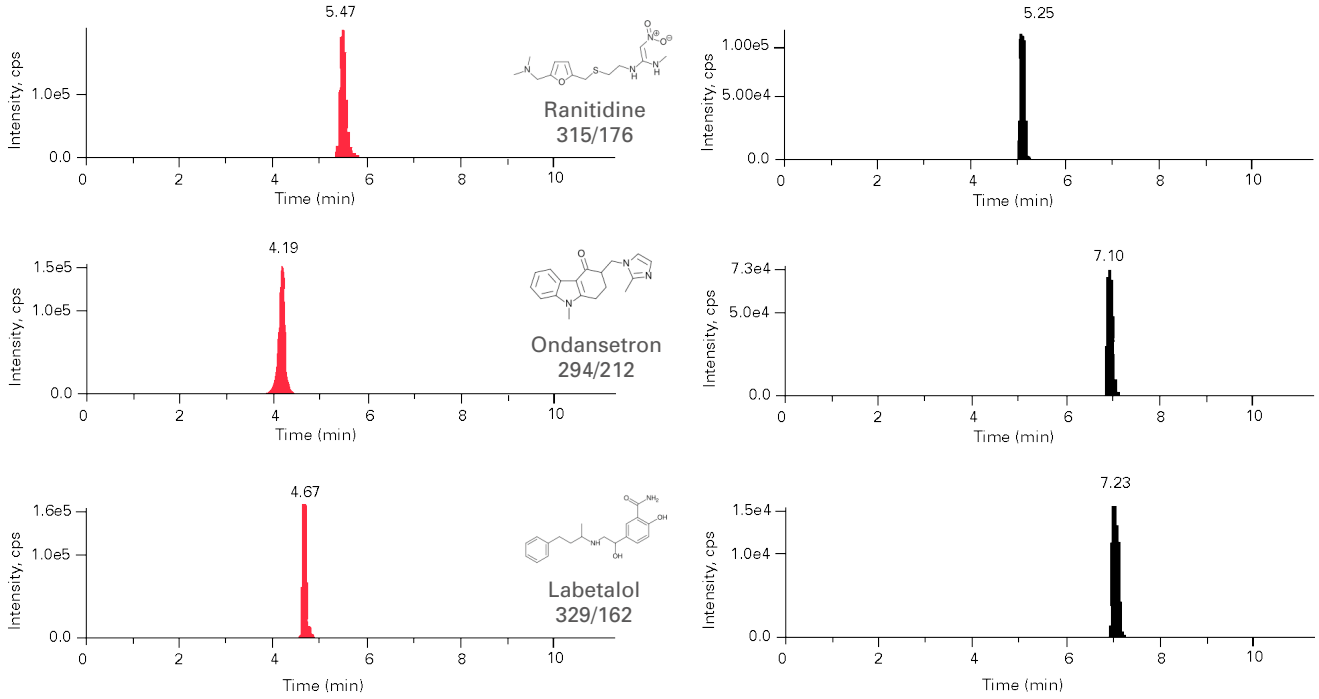

Figure 2

Column:	TSKgel Amide-80 3 µm (2.0 mm ID x 15 cm L)
Eluent :	A: 10 mM Ammoniumformiate (pH 3.75) B: ACN
Gradient :	0 min (B 90%) -> 10 min (B 40%) ->13 min (B 40%)
Flow rate :	0.2 mL/min
Inj. volume :	5 µL (50 µg/L)
Detection :	QTrap® LC-MS/MS (Applied Biosystems), ESI+

Column: :	TSKgel ODS-100V 3 µm (2.0 mm ID x 15 cm L)
Eluent :	A: 10 mM Ammoniumformiate (pH 3.75) B: ACN
Gradient :	0 min (B 0%) -> 10 min (B 80%) ->13 min (B 80%)
Flow rate :	0.2 mL/min
Inj. volume :	5 µL (50 µg/L)
Detection :	QTrap® LC-MS/MS (Applied Biosystems), ESI+

The elution order is usually inversed as well and polar compounds are very well separated. The portion of organic solvent in the mobile phase is relatively high supporting highly sensitive LC–MS detection.
We present examples for the different elution order of polar compounds in HILIC and RPC with UV detection and with LC–MS–MS detection.

HILIC AND REVERSED PHASE SEPARATION OF PEPTIDES

The selectivity of HILIC is completely different from RPC and for most samples the elution order is inversed. Figure 1 gives an example for these differences in selectivity. In addition it shows that HILIC can be applied not only for the separation of saccharides or polyols but for peptides as well.
Small peptides were separated by C18 and HILIC columns of the same dimensions. Mobile phases - acetonitrile and 0.1% trifluoroacetic acid - were the same for both separations but gradients were almost inversed. The very polar peptide 8 (DSDPR), which was not separated from the shorter peptides 4 and 5 in RP mode was highly retained in HILIC mode. Elution order for peptides 2 and 3 was inversed.

HILIC–MS ANALYSIS OF POLAR DRUG SUBSTANCES

HPLC–MS–MS has become a powerful tool for analysis of drug substances. It is used in quantitative confirmatory analysis of drugs of abuse, as well as in fast analysis of metabolites in pharmacokinetics or rapid screening of drug candidates.
Analysing basic drug substances by RPC has some drawbacks: In addition to peak tailing problems, very polar components show low retention and elute with high water containing eluents, which are unfavourable for subsequent ESI–MS detection. The new 3 µm particle size TSKgel Amide-80 columns were designed to provide HILIC columns for LC–MS use. They combine both, retention for polar compounds and high resolution. An additional benefit of TSKgel Amide-80 for mass spectrometric detection is the virtual absence of column bleeding.

Figure 2 shows the analysis of some basic drug substances using a HILIC column (TSKgel Amide-80 3 µm) compared to the analysis of the same molecules using a RP column (TSKgel ODS-100V 3 µm). Ranitidine, a histamine H2-receptor antagonist, ondansetron, anantiemetic serotonin receptor antagonist, and labetalol, an alpha-1 and beta adrenergic blocker, used to treat high blood pressure, were selected to demonstrate the differences in selectivity and MS-signal

response when using different chromatographic modes. Ranitidine, which has the highest number of polar groups among these molecules, showed the highest retention on the HILIC phase and the lowest retention on the RPC phase. The signal intensity was almost doubled for ranitidine when using HILIC separation mode compared to RPC. The example of labetalol shows that even a ten-fold increase in signal height can be achieved by using HILIC instead of RPC.

CONCLUSION

TSKgel Amide-80 HILIC columns with 5 µm particles have been used for years for a broad range of LC–MS applications[4,5]. The new 3 µm Amide-80 columns provide even better resolution at reduced analysis time as a result of the smaller particle size. The unique HILIC separation mechanism offers major advantages for the analysis of very polar compounds by LC–MS techniques.

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5. H. Nakagawa et al., J. Chromatogr. B, 853(1–2), 133–137 (2007).

Separation of Polar Molecules using a Stable Amino-bonded Phase HILIC Column

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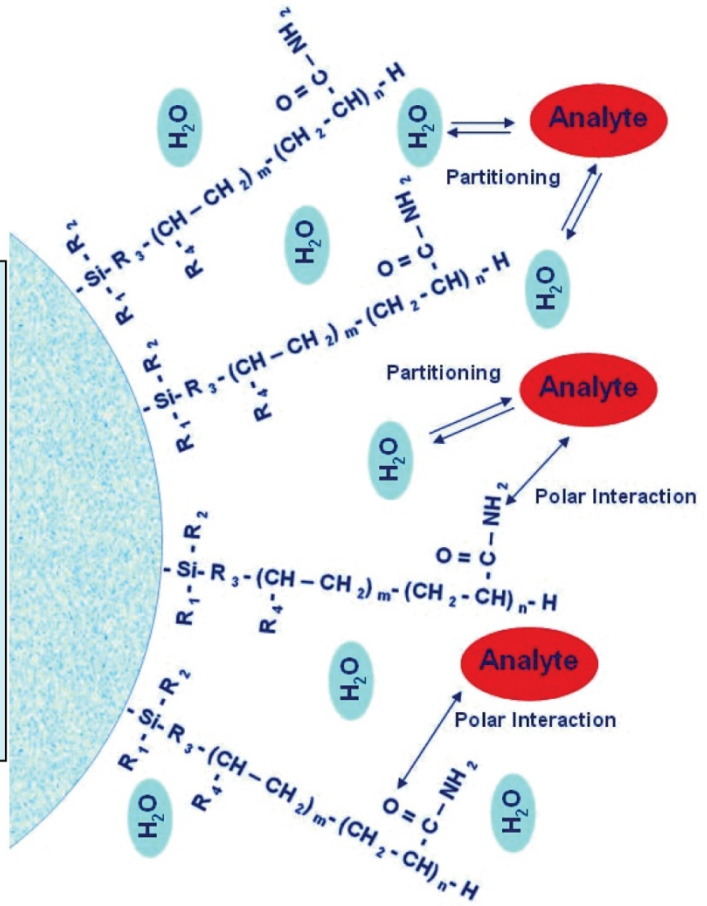
Introduction

- 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.
- 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 ammonium acetate buffer.
- In contrast to the retention behavior in reversed phase, in HILIC, solutes will be retained longer when increasing the percent acetonitrile.

Structure and Mechanism

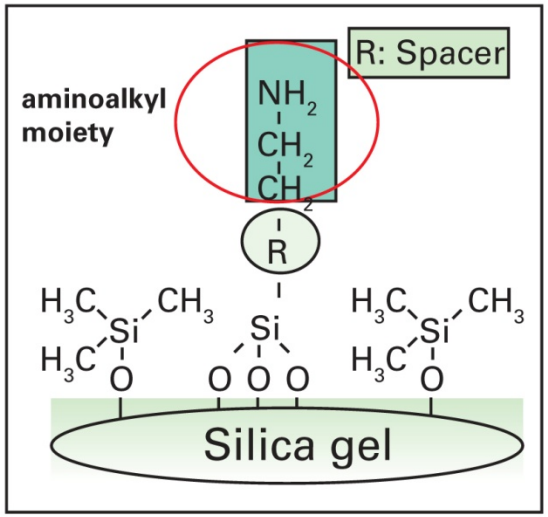
- Polar stationary phase as in normal phase LC
- Mobile phase similar to reversed phase (high organic)
- Elution in order of increasing hydrophylicity



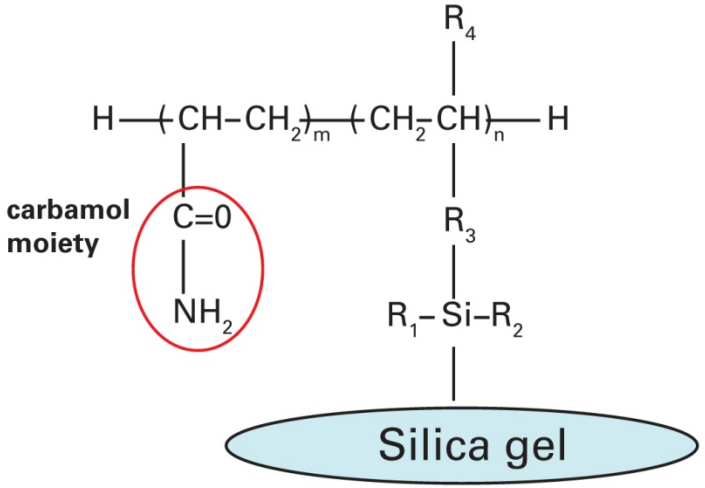
Mechanism of Hydrophilic Interaction Liquid Chromatography (HILIC)

Introduction

Structure of TSKgel® NH_2-100



Structure of TSKgel Amide-80



TSKgel NH_2-100	
Particle size (µm)	3
Pore size (nm)	10
Surface area (m^2/g)	450
Functionality	**aminoalkyl**

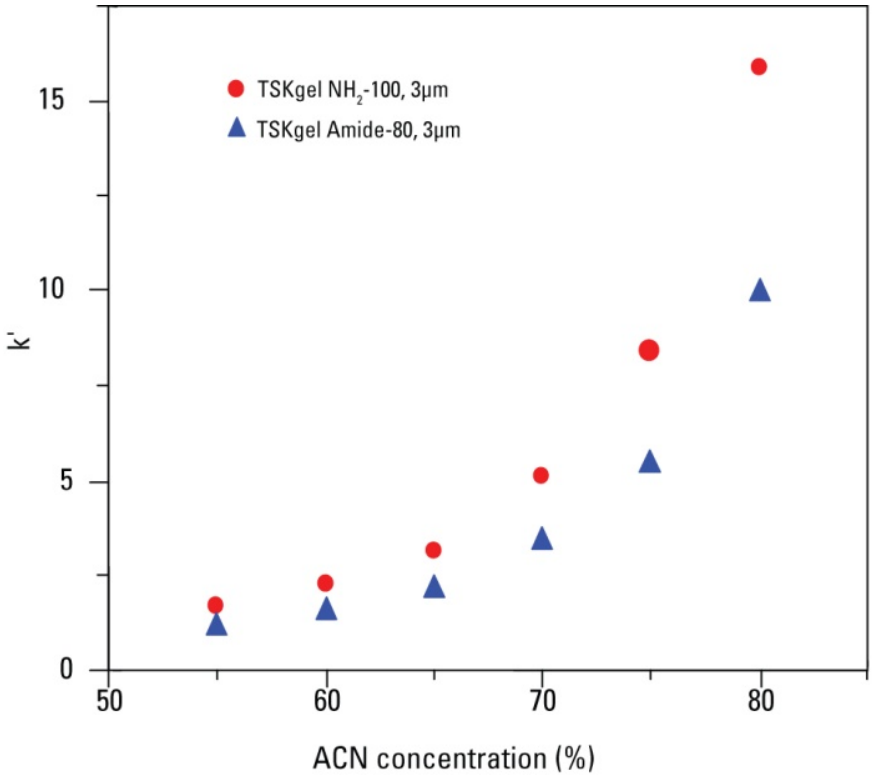
TSKgel Amide-80	
Particle size (µm)	3
Pore size (nm)	10
Surface area (m^2/g)	450
Functionality	Carbamoyl group

TSKgel Amide-80 and NH_2-100 Columns were designed for HILIC

Both can be used with evaporative light scattering (ELS) and mass spec (MS) detectors.

The 3µm material is ideal for use in LC/MS applications for the analysis of active pharmaceutical ingredients and their metabolites.

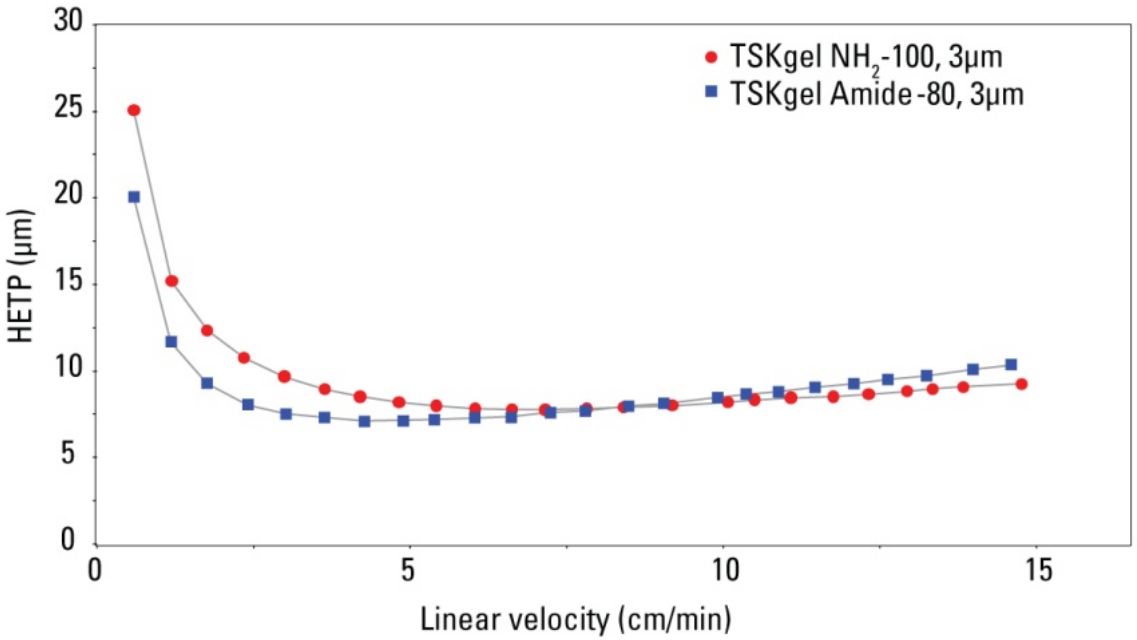
Retention of TSKgel HILIC Columns



Columns:	TSKgel NH_2-100, 3µm, 4.6mm ID x 15cm
	TSKgel Amide-80, 3µm, 4.6mm ID x 15cm
Mobile phase:	H_2O/ACN = 10/90
Flow rate:	1.0mL/min
Detection:	RI
Temperature:	40°C
Injection Vol.:	10µL
Sample:	inositol

Amino-based TSKgel NH_2-100 columns expand retention & selectivity in HILIC while offering higher chemical stability, a pre-requisite for reproducible results.

Column Efficiency

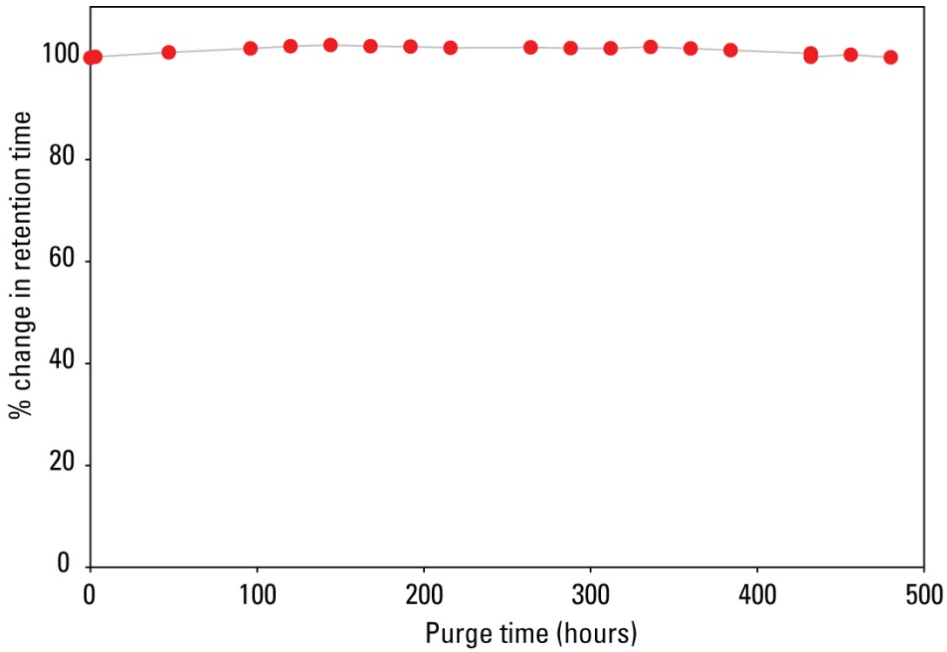


TSKgel NH_2-100, 3µm, 4.6mm ID x 15cm
TSKgel Amide-80, 3µm, 4.6mm ID x 15cm

Mobile phase:	H_2O/ACN = 10/90
Flow Rate:	0.1 ~ 2.4mL/min
Detection:	UV@254nm
Temperature:	40°C
Injection vol.:	10µL
Sample:	uracil

As expected, HETP vs. Linear Velocity is similar for both columns, since the TSKgel NH_2-100 and Amide-80 columns are prepared from the same spherical 3µm silica particles.

Column Stability



Column:	TSKgel NH_2-100, 3µm, 4.6mm ID x 5cm
Mobile phase:	H_2O/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 barely changed.

Applications

- Here we report the separation of a variety of polar molecules using a stable amino-bonded phase HILIC column.
- We have also reported the separation of polar compounds using an carbamoyl (amide) bonded phase HILIC column.
- Organic acids are widely used in different food and beverages.
- 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.
- The analysis of saccharides provides valuable information for the medical, research and food industries.

Introduction

- In the past various analytical techniques have been used to analyze saccharides, including all modes of high performance liquid chromatography (HPLC).
- Normal phase chromatography, in tandem with a differential refractometer as a detector, has long been used 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, while most of the substances with low polarity, as well as monohydric alcohols, elute in the void or very close to the void volume of the column.
- Separation is valuable in method development and in quality control for the identification and quantification of these compounds.

Objective

To show the usefulness of the silica-based TSKgel NH_2-100 and TSKgel Amide-80 HILIC columns for analysis of different types of polar molecules using a conventional HPLC system.

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 (organic acids):

- Column: TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm
- Detection: UV@210nm
- Column temp: 40˚C
- Flow rate: 0.2mL/min
- Injection vol.: 5µL
- Mobile phase (Isocratic): 70% ACN:30% 5mmol/L ammonium acetate in H_2O, pH 4.1

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
- Column temp: 50°C
- Flow rate: 0.2mL/min
- Injection vol.: 2µL
- Mobile phase (Isocratic): 80% ACN in H_2O

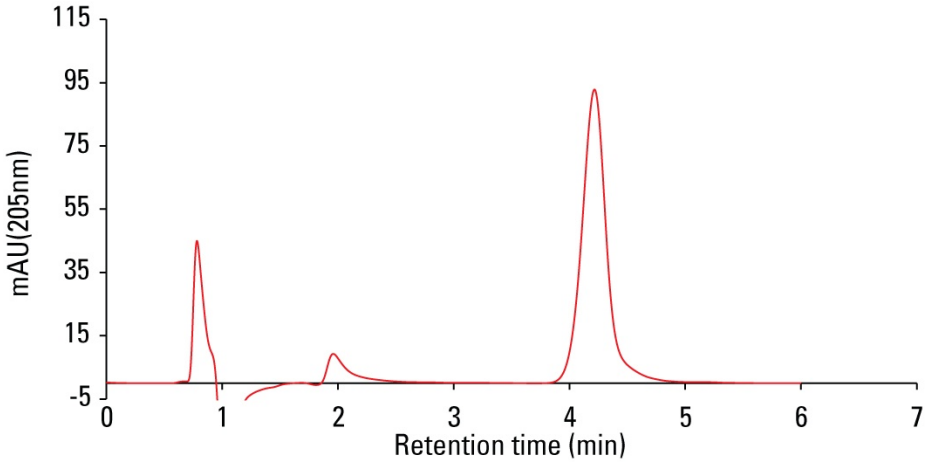
Material and Methods (contd.)

All the standards and samples were pure analytical grade from Sigma Aldrich.

All the standards and samples were filtered through a 0.45µm filter before injecting onto the column.

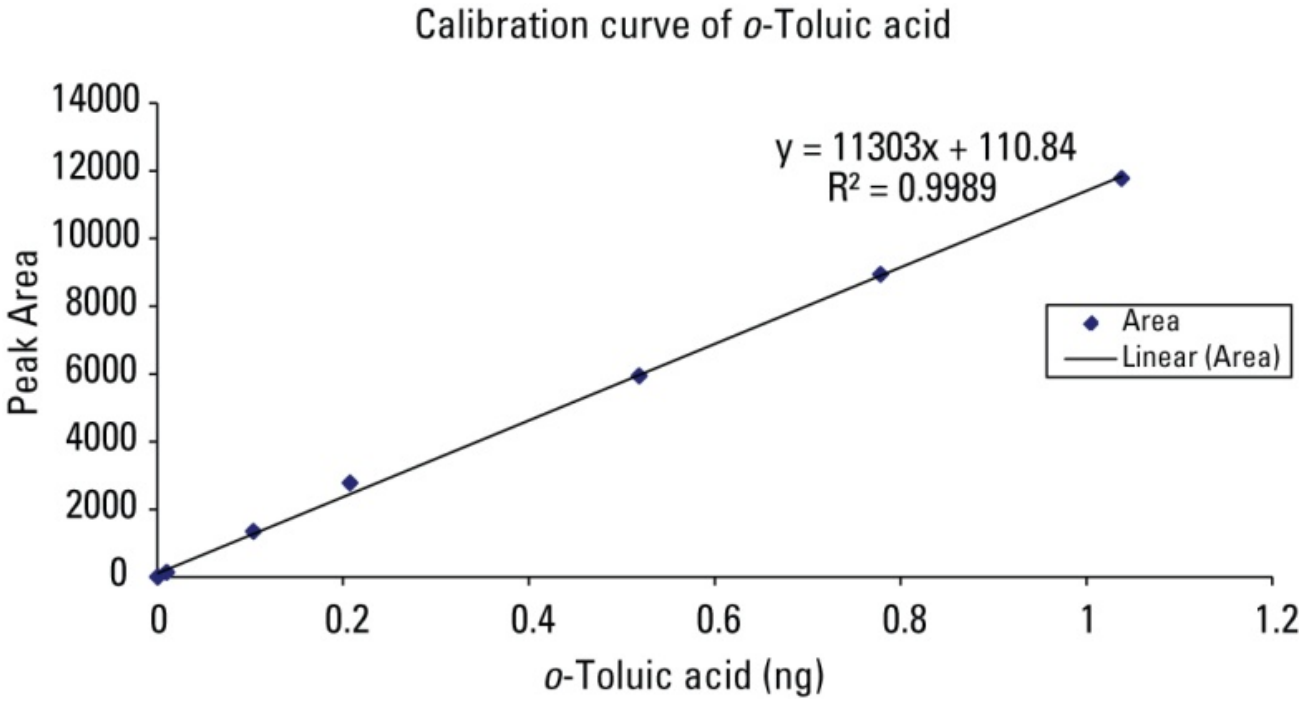
High purity chemicals and HPLC grade solvents were used for the preparation of stock standards, samples and mobile phases.

Analysis of *o*-Toluic Acid using a TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm Column



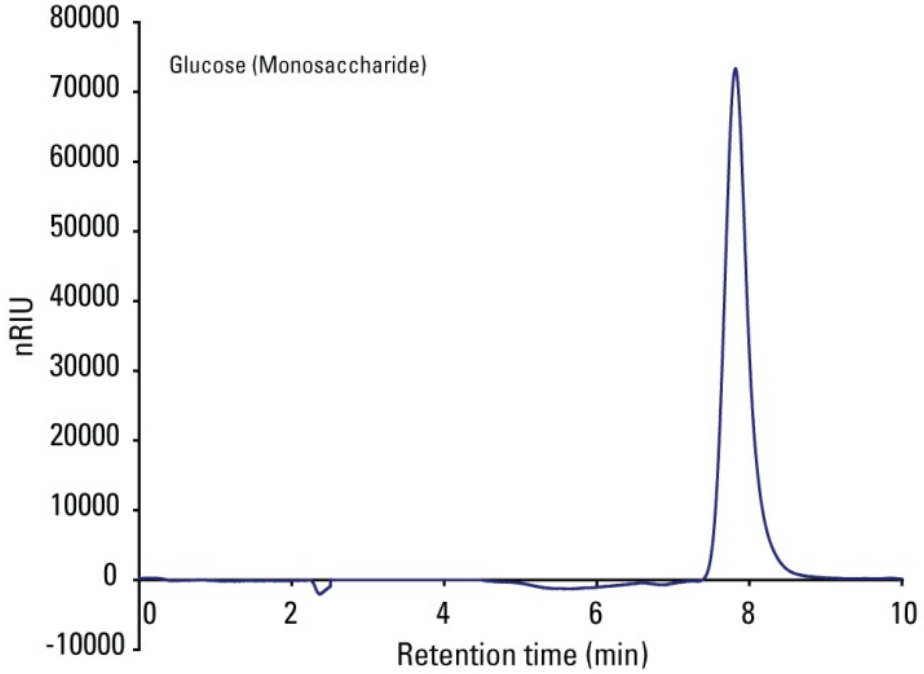
Column:	TSKgel NH_2-100, 3um, 2.0mm ID x 5cm
Mobile phase:	70% ACN:30% 5mmol/L in ammonium acetate in H_20, pH 4.1
Flow rate:	0.2mL/min
Detection:	UV@210nm
Temperature:	40°C
Injection vol.:	5µL
Sample:	*o*-toluic acid

Loading Capacity



- **The coefficient of linear regression for the calibration curve of *o*-toluic acid was 0.9989 over the concentration range of 0.01-1ng.**
- **Similarly other organic acids viz. p-amino benzoic acid, p-toluene-sulfonic acid, benzoic acid using this column.**
- **The limit of detection of sorbic acid was 51ppm.**
- **5-fluoro uracil also could be retained using this column.**

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

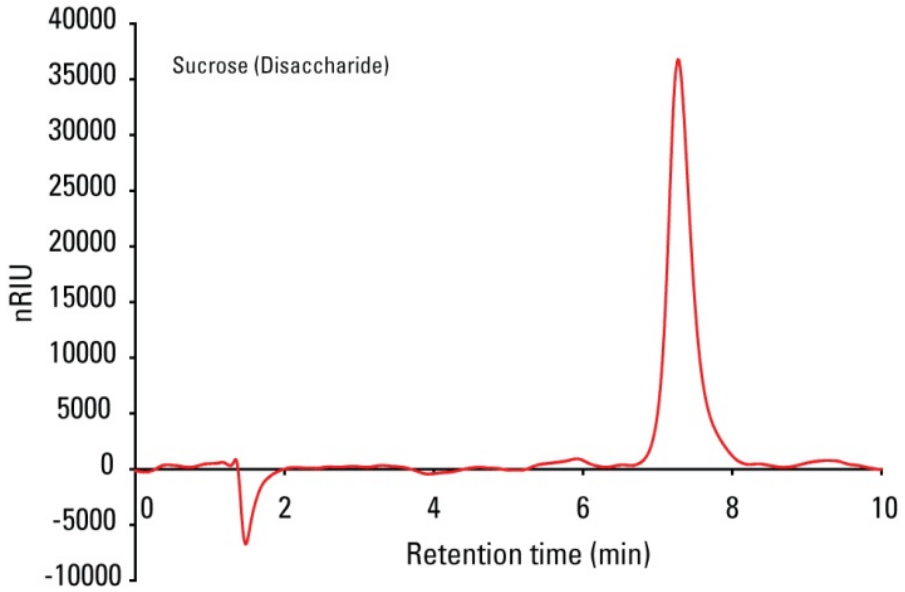


Columns:	TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm
Mobile phase:	80% ACN in H_2O
Flow rate:	0.2mL/min
Detection:	RI
Temperature:	50°C
Injection Vol.:	2µL

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

Analysis of Sucrose (disaccharide) 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

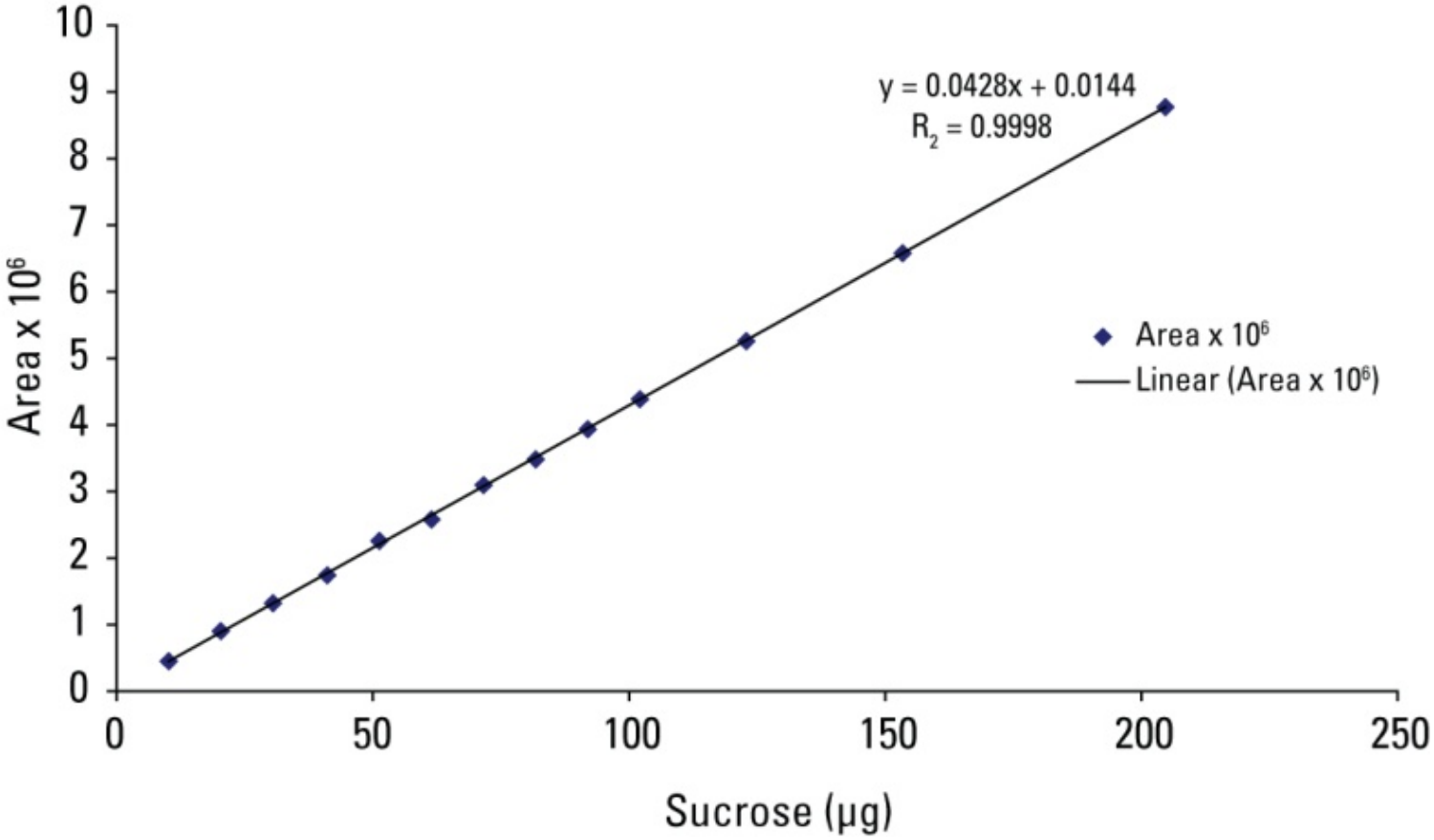
System Suitability Study

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

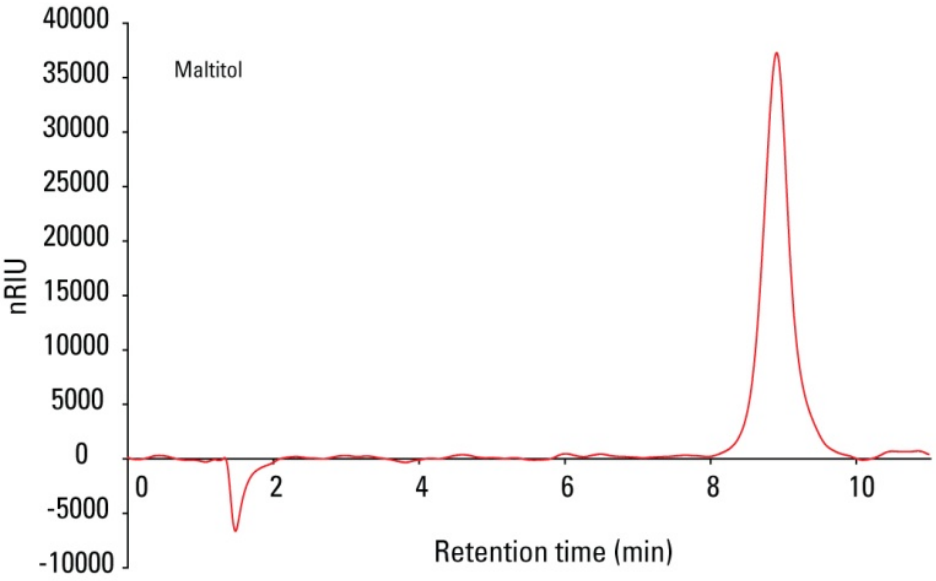
Three consecutive injections of sucrose yielded very consistent results for all peak parameters that determine the suitability of the system and method.

Loading Capacity



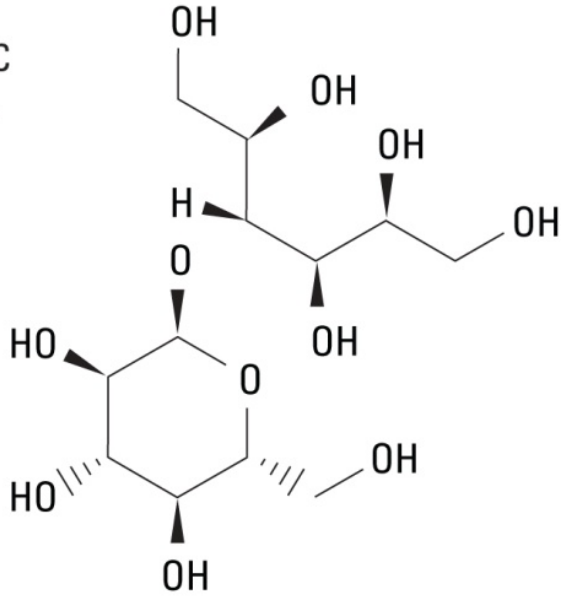
Sucrose can be analyzed with a high degree of linearity over the experimental concentration range shown in this figure.

Analysis of Maltitol (polyol or sugar alcohol) using a TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm Column

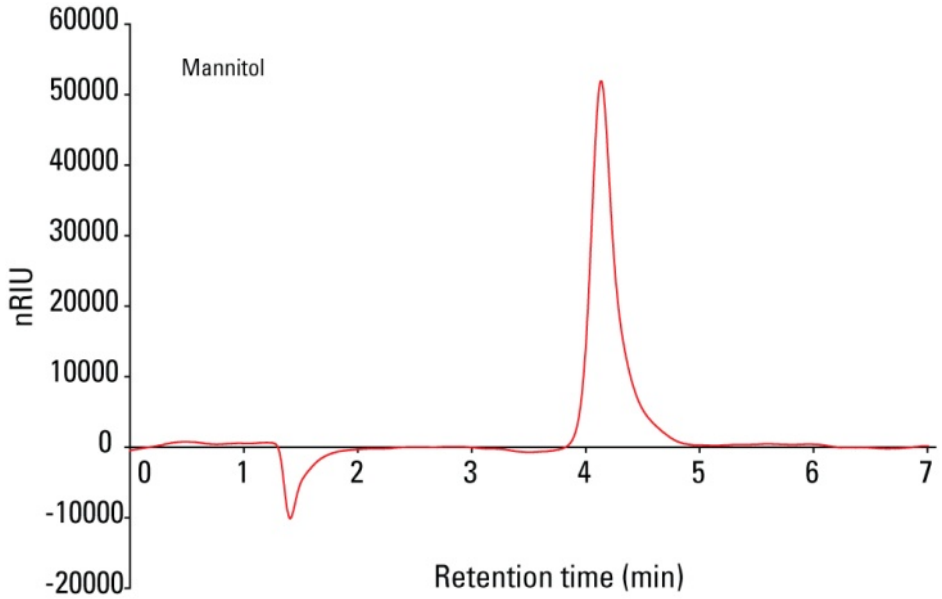


RT	k	Area x 10^6	A_s	Plates
8.908	13.18	104	1.05	3019

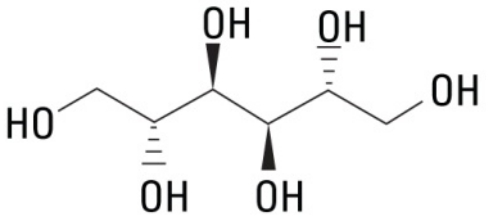
Column:	TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm
Mobile phase:	80% ACN in H_20
Flow rate:	0.2mL/min
Detection:	RI
Temperature:	50°C
Injection vol.:	2µL



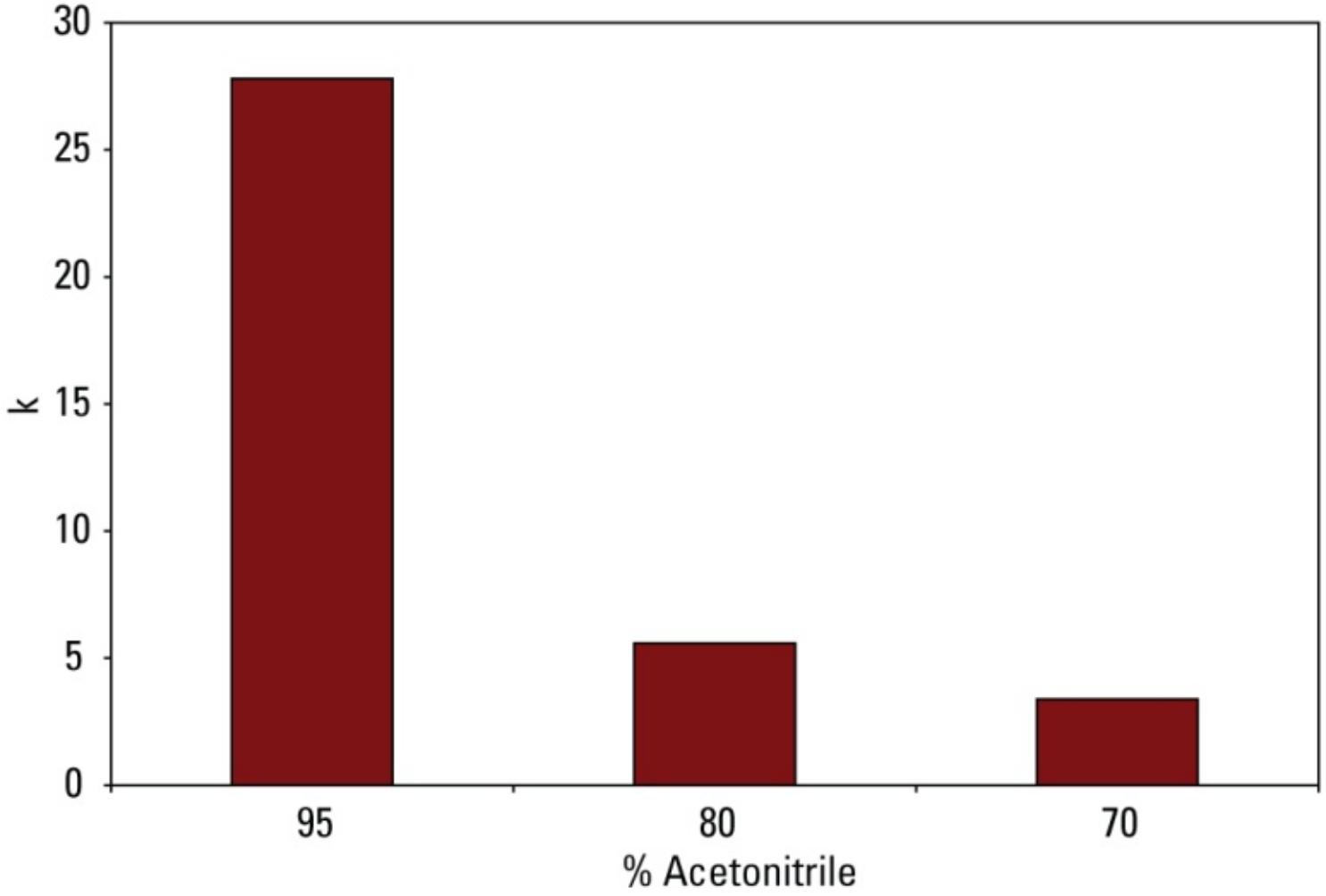
Analysis of Mannitol (polyol or sugar alcohol) using a TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm Column



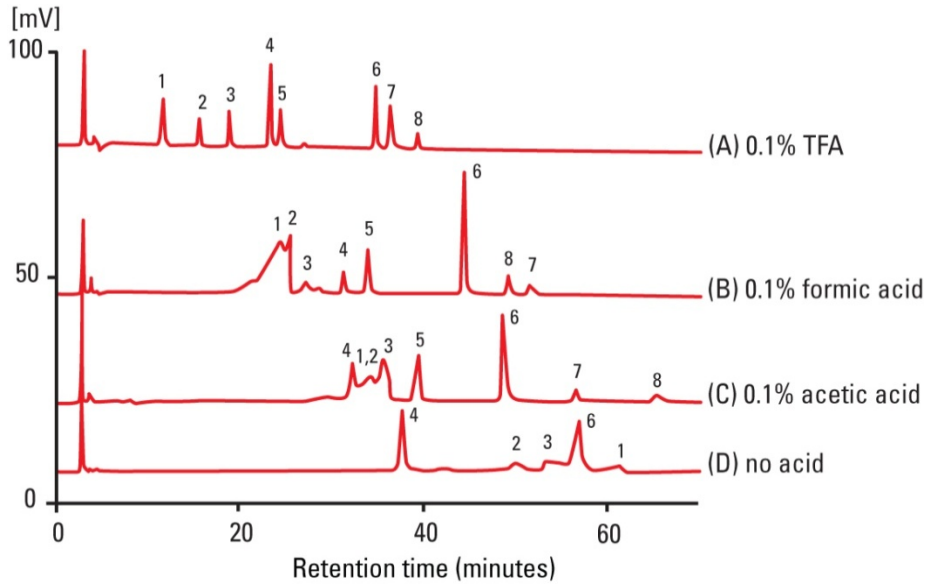
Column:	TSKgel NH_2-100, 3um, 2.0mm ID x 5cm
Mobile phase:	80% ACN in H_20
Flow rate:	0.2mL/min
Detection:	RI
Temperature:	50°C
Injection vol.:	2µL



Effect of Acetonitrile Concentration on the Retention of Mannitol using a TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm Column



Separation of Peptides by HILIC using a TSKgel Amide-80 Column

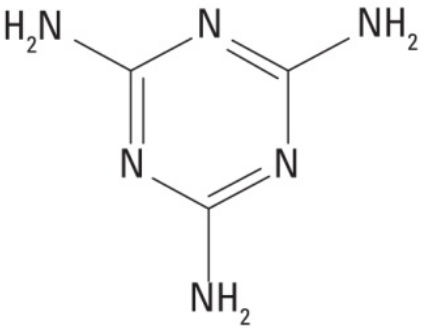


Peak/Peptide Number	Sequence	Recovery from TSKgel Amide-80	(%) Recovery from TSKgel ODS-80T$_S$*
1	FY	96	96
2	FGGF	101	89
3	FLEEI	98	93
4	DYMGWMDP-NH2	90	74
5	NFTYGGF	90	95
6	AGSQ	96	65
7	WAGGDASGE	85	96
8	YGGFMTSQKSQTPLVT	92	96
9	ASTTTNYT	94	89
10	VLSEGEWQLVLHVW AKVEADVAGHGQDI LIRLFKSHPETLEKFD RFKHLKTEAEM	80	62

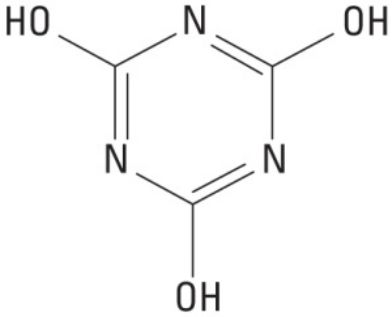
*TSKgel ODS-80T$_S$ run was at 83.3 min. linear gradient of ACN from 5 to 55% in 0.1% TFA

Simultaneous Determination of Melamine and Cyanuric Acid by HILIC MS/MS using a 3µm TSKgel Amide-80 Column

Structural formulas of melamine and cyanuric acid



Melamine



Cyanuric acid

Pretreatment of milk

Milk + H_2O/ACN = 20/80 = 10 + 90 (v/v)

↓

Mix

↓

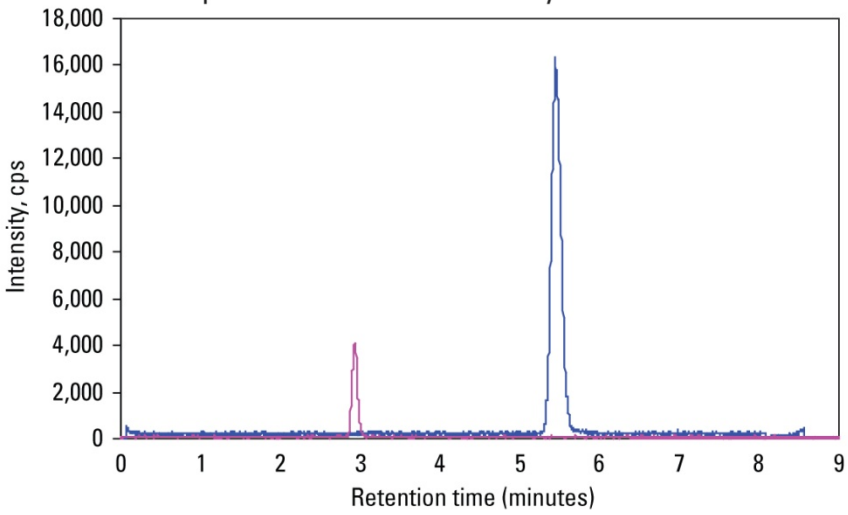
Ultracentrifugation @ 5,000rpm for 5minutes

↓

Filtration (pore size: 0.5µm)

Simultaneous Determination of Melamine and Cyanuric Acid by HILIC MS/MS using a 3µm TSKgel Amide-80 Column

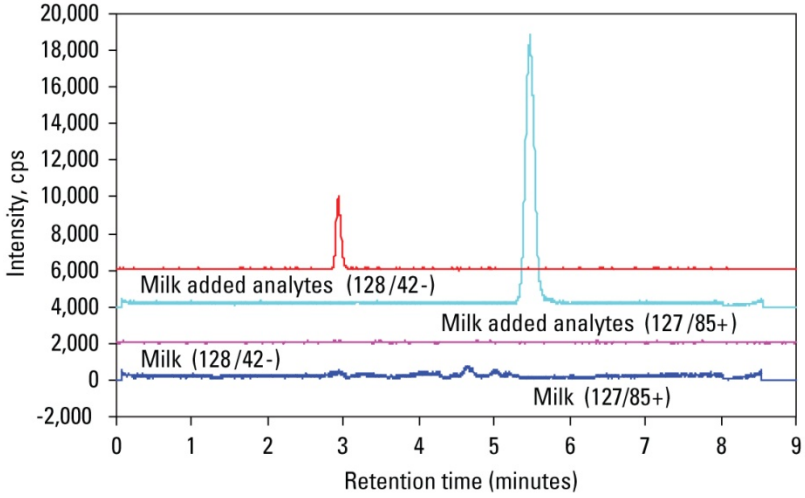
Separation of melamine and cyanuric acid in milk



TSKgel Amide-80, 3µm, 2.0mm ID x 15cm

Eluent:	A: 0.05% formic acid in H_2O
	B: 0.05% formic acid in ACN
	A/B=25/75
Flow rate:	0.2mL/min
Temperature:	40°C
Injection vol.:	5µL
Instrument:	QTRAP® (AB Sciex)
Ion source:	ESI
	127/85+ (melamine)
	128/42- (cyanuric acid)

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

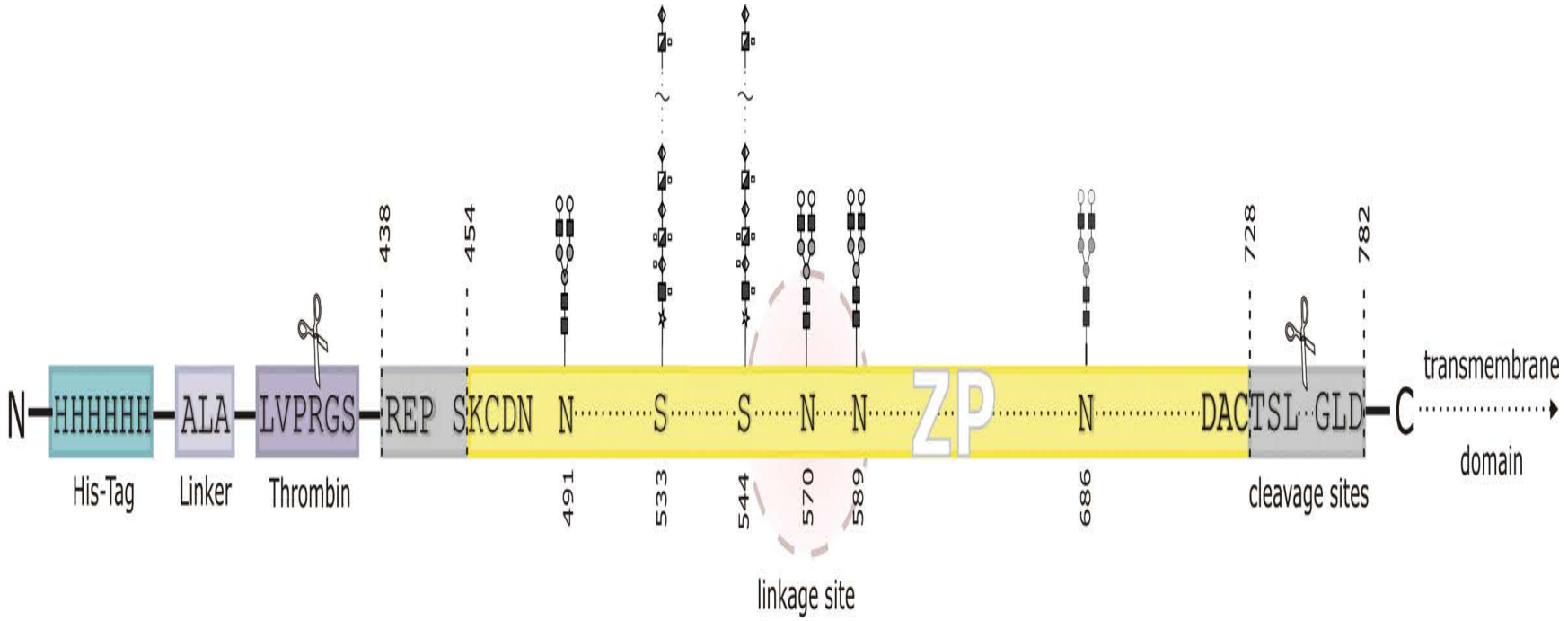


TSKgel Amide-80, 3µm, 2.0mm ID x 15cm

Eluent:	A: 0.05% formic acid in H_2O
	B: 0.05% formic acid in ACN
	A/B=25/75
Flow rate:	0.2mL/min
Temperature:	40°C
Injection vol.:	5µL
Instrument:	QTRAP (AB Sciex)
Ion source:	ESI
	127/85+ (melamine)
	128/42- (cyanuric acid)

Identification of Isobaric Glycoforms by Retention Time (Glycobase) and MS/MS Experiments

Protein construct of the zp domain of murine tgfr-3 expressed in HEK293EBNA



Separations of 2-AB Labeled N-glycans

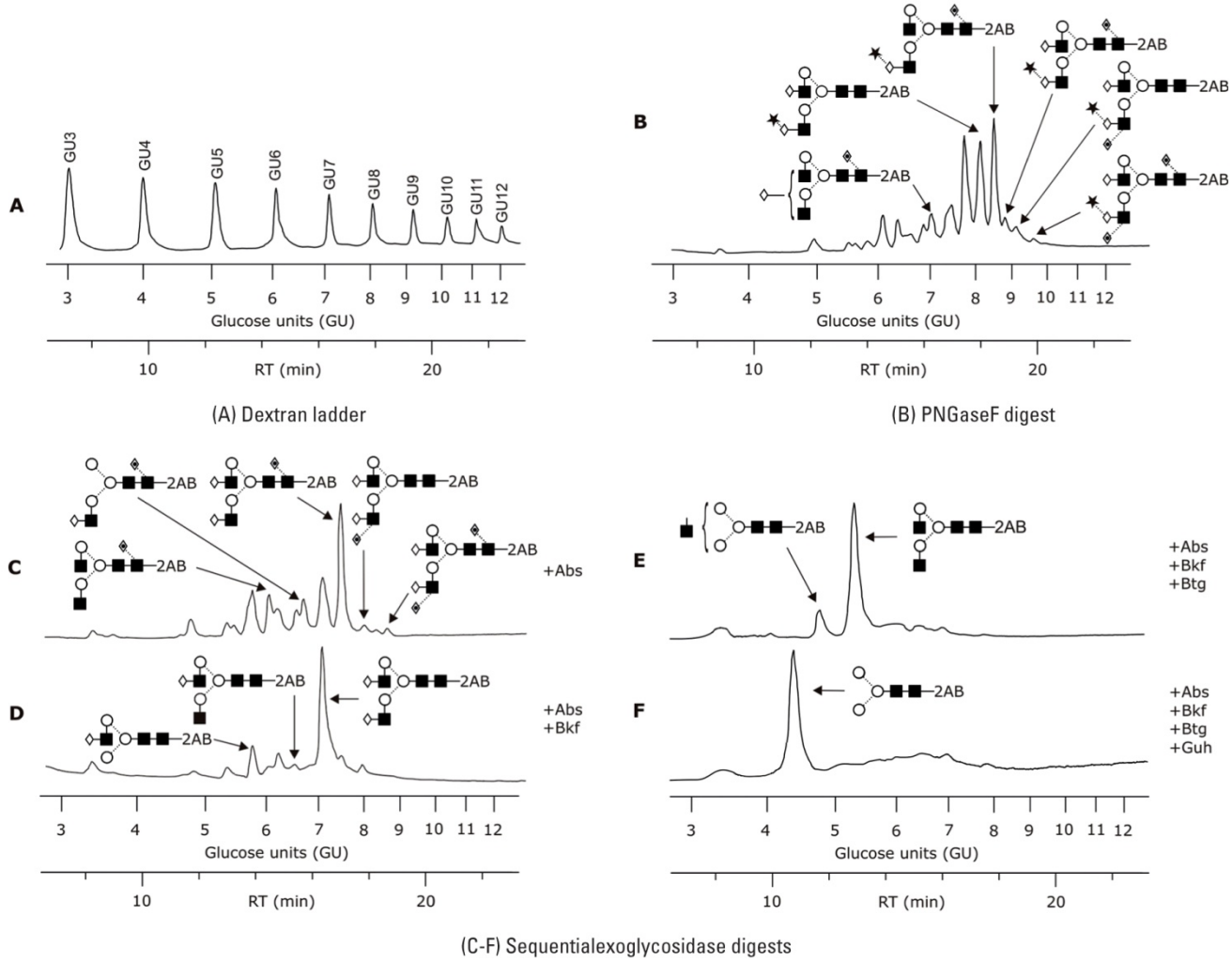
Fluorescence chromatograms of HILIC separations of 2-AB labeled N-glycans released from the recombinant ZP domain construct of murine TGFR-3, were compared to the dextran ladder.

Chromatographic Parameters

Column:	TSKgel Amide-80, 3µm, 2mm ID x 15cm
Mobile phase:	A: 50mmol/L ammonium formate, pH 4.3
	B: acetonitrile
Gradient:	0-35 min: 75-35% B
Flow rate:	0.22mL/min
Detection:	Fluorescence; excitation @ 360nm, emission @ 425nm
Temperature:	50°C
Injection vol.:	2µL, approximately 300fmol for GU3

The structural analysis was completed by high resolution mass spectra acquired on a MALDI QIT TOF MS instrument.

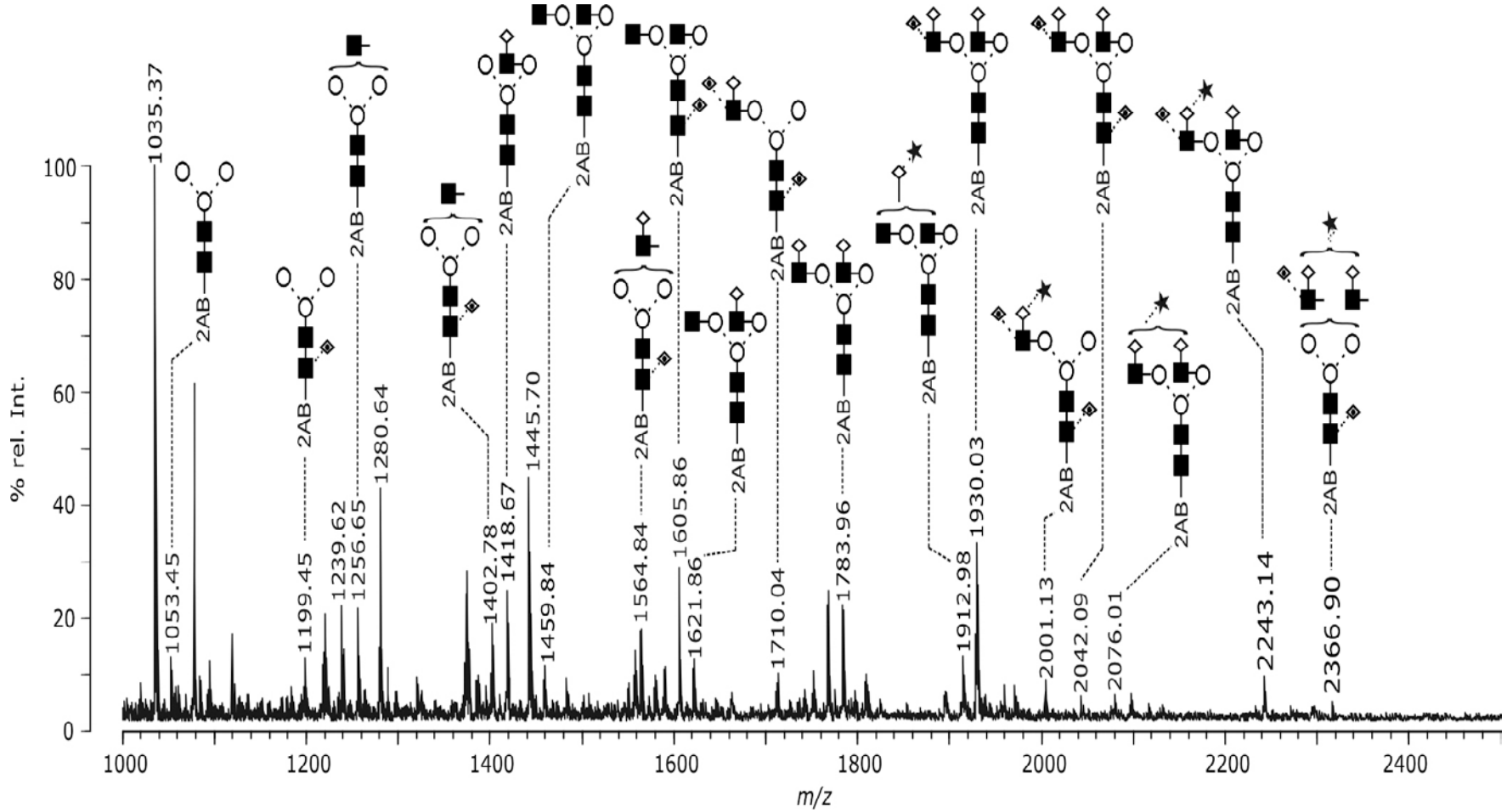
Separations of 2-AB Labeled N-glycans



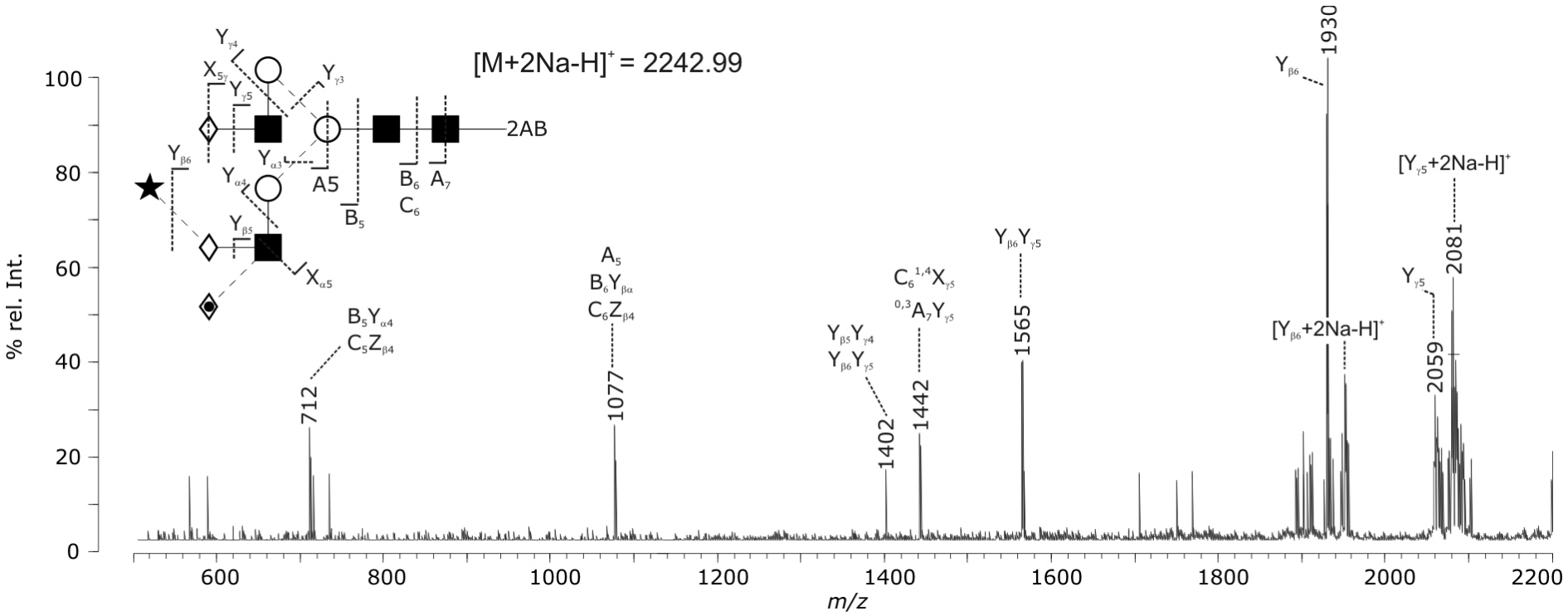
Used exoglycosidase:

- Sialidase A (Abs)
- α-Fucosidase (Bkf)
- β-Galactosidase (Btg)
- β-N-Acetylhexoamidase (Guh)

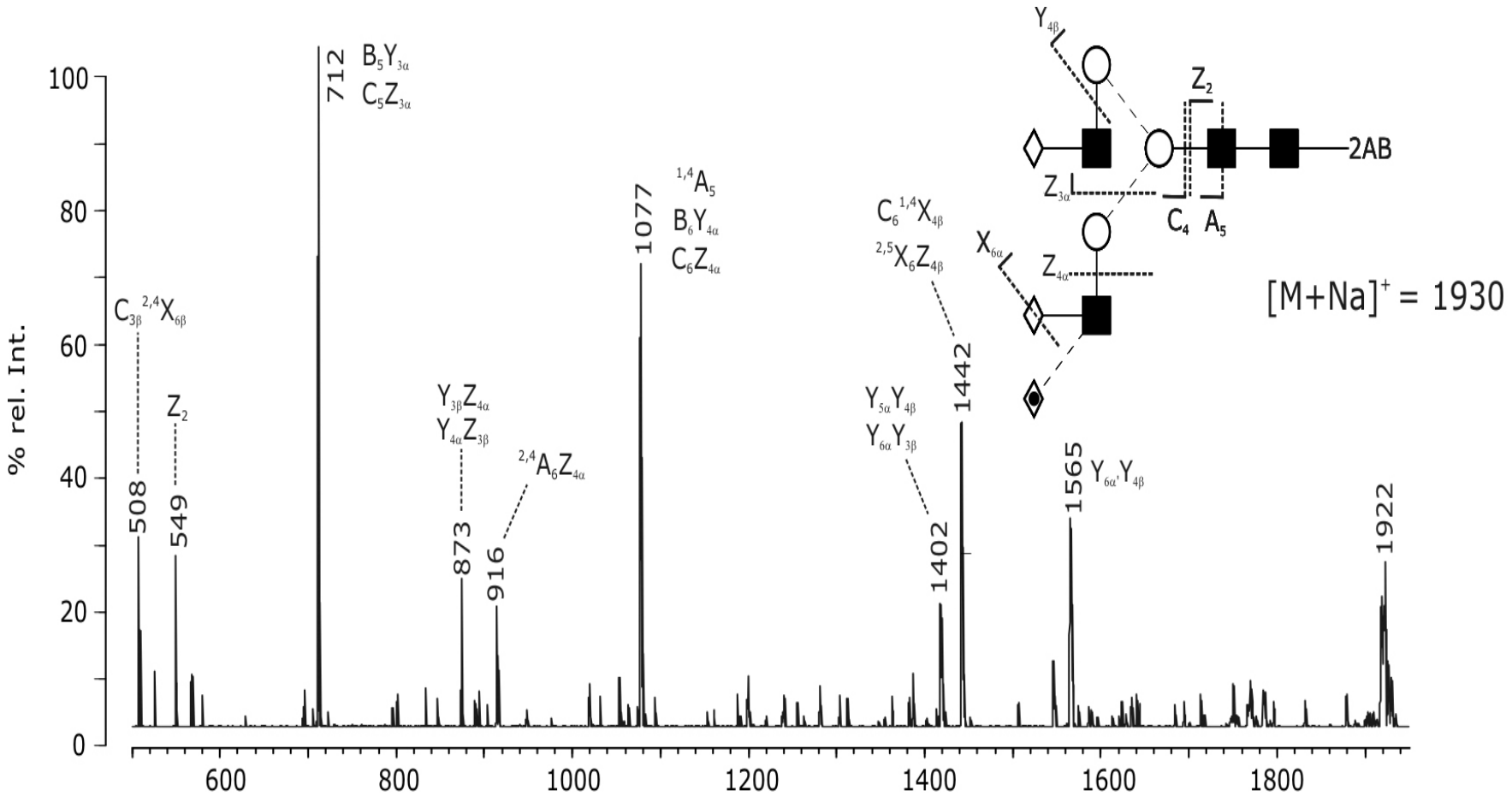
MALDI Mass Spectrum of 2-AB-labeled Glycans Released from ZP domain Construct of Murine TGFR3



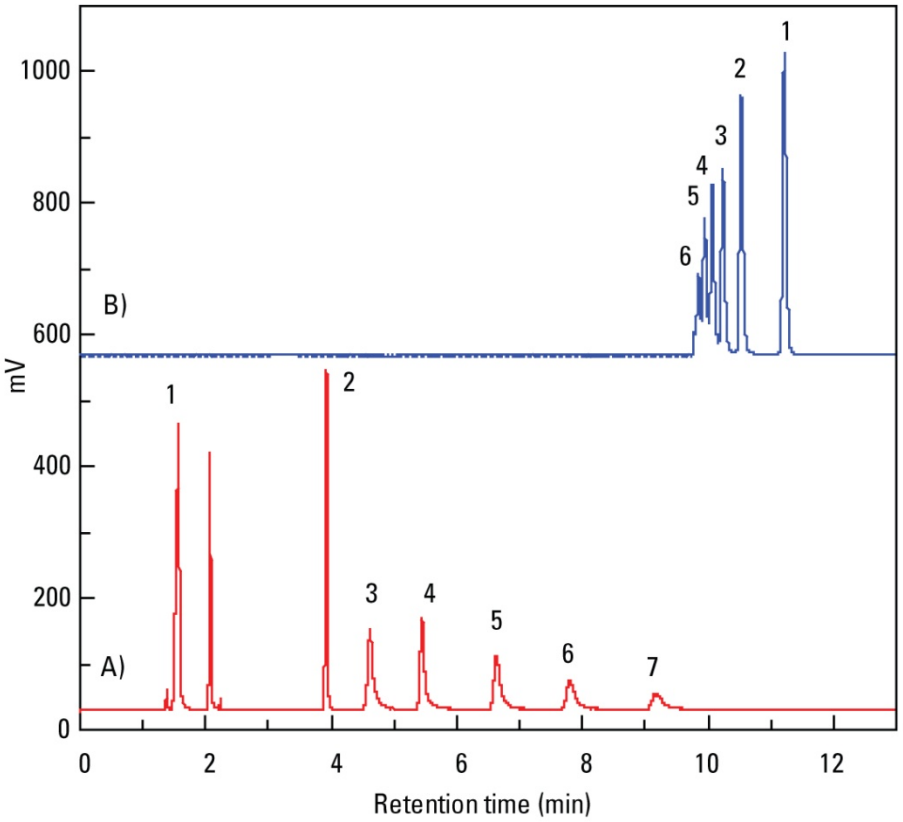
MS2 (CID) Mass Spectrum of m/z 2243



MS3 (CID) Mass Spectrum of m/z 1930



Comparison of Chromatograms of MTX and its Derivatives



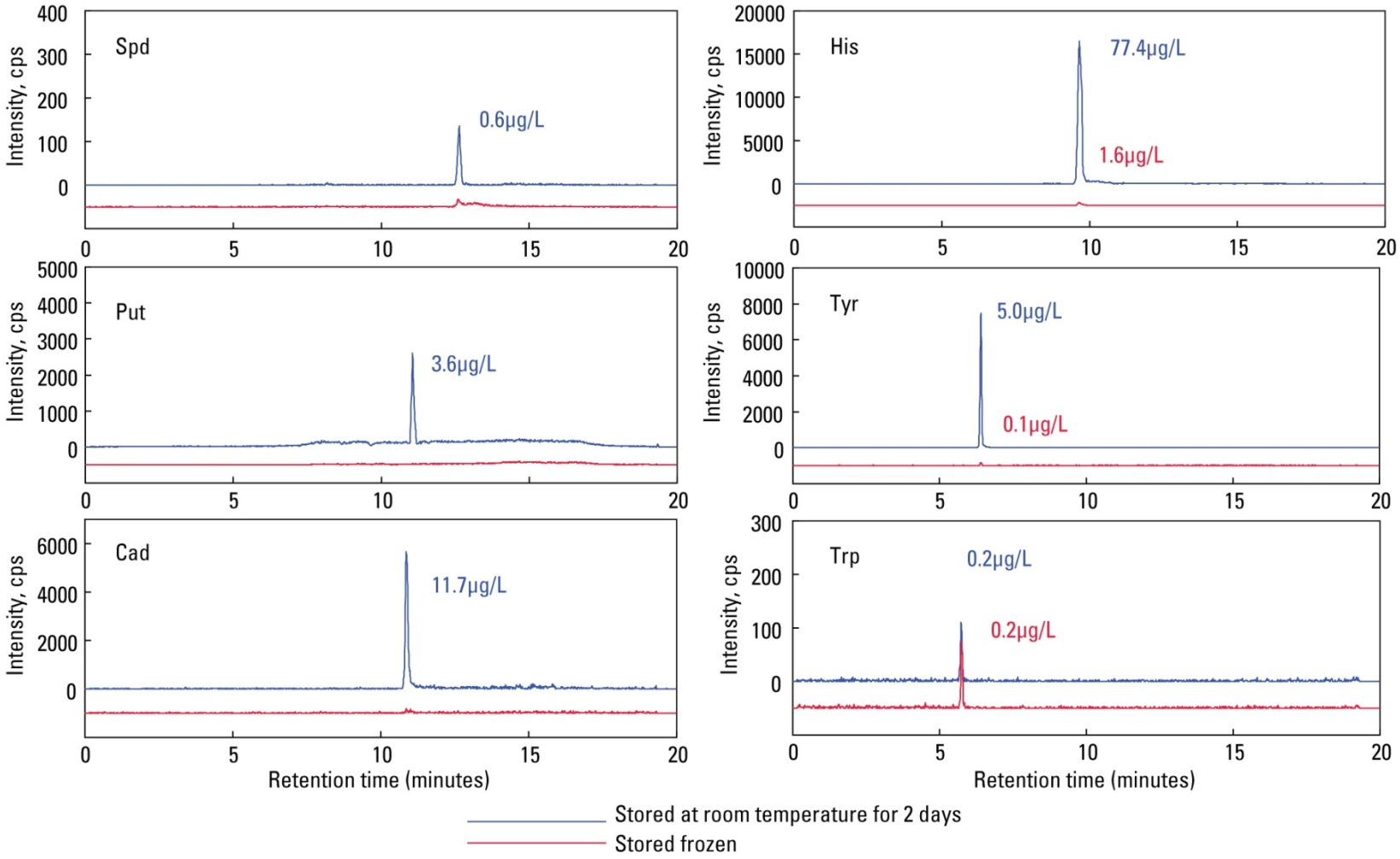
Columns:	A) TSKgel NH_2-100, 3µm, 2.0mm ID x 15cm
	B) TSKgel ODS-100V, 3µm, 2.0mm ID x 15cm
Mobile phase:	A: A) H_2O/ACN (10/90) + 0.1% TFA
	B) H_2O + 0.1% TFA
	B: A) H_2O/ACN (10/90) + 0.1% TFA
	B) ACN + 0.1% TFA
Gradient:	0% B (0min), 40% B (15min), 0% B (17min)
Flow rate:	0.20mL/min
Detection:	UV@313nm
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. $MTXPG_7$

Biogenic Amines in Tuna as Function of Storage

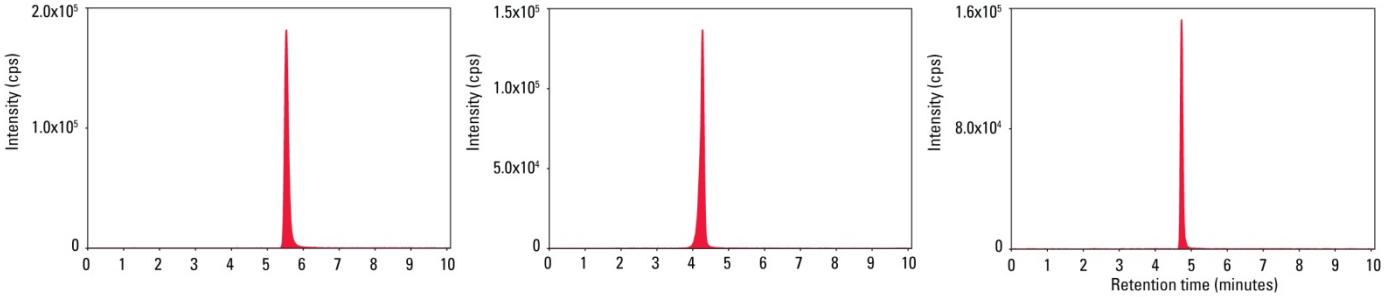
Analytical Conditions of LC/MS/MS

LC System:	Agilent 1200SL Series
Column:	TSKgel Amide-80, 3µm, 2.0mm ID x 15cm
Mobile phase:	A: 30mmol/L ammonium formate in H_2O, pH 4.0
	B: ACN
Gradient:	0min (90%B), 12min (40%B), 14min (40%B), 16min (90%B)
Flow rate:	0.2mL/min
Temperature:	50°C
Injection vol.:	2µL
MS: QTRAP®	(AB SCIEX)
Ion source:	ESI
Polarity:	Positive
Mode:	MRM
Precursor ion/Product ion:	
Spermidine (Spd):	146.3/72.1
Putrescine (Put):	89.1/72.1
Cadaverine (Cad):	103.1/86.1
Histamine (His):	112.0/95.0
Tyramine (Tyr):	138.0/121.0
Tryptamine (Trp):	161.0/115.0

Biogenic Amines in Tuna as Function of Storage

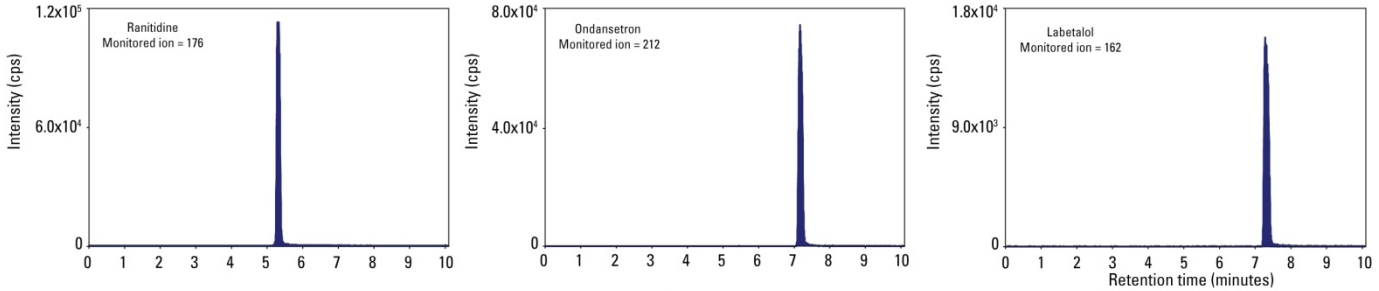


LC/MS/MS Analysis of Polar Basic Drugs - HILIC or RPC Mode?



TSKgel Amide-80, 3µm, 2.0mm ID x 15cm

Mobile phase :	A: 10mol/L ammonium formate, pH 3.75 B: ACN
Gradient :	0min (90%B), 10min (40%B), 13min (40%B)
Flow rate :	0.2mL/min
Injection vol.:	5µL (50µg/L)
Instrument :	QTRAP LC/MS/MS, ESI+



TSKgel ODS-100V, 3µm, 2.0mm ID x 15cm

Mobile phase :	A: 10mol/L ammonium formate, pH 3.75 B: ACN
Gradient :	0min (90%B), 10min (40%B), 13min (40%B)
Flow rate :	0.2mL/min
Injection vol.:	5µL (50µg/L)
Instrument :	QTRAP LC/MS/MS, ESI+

Due to the high organic content of the eluent, HILIC analysis provides increased detection sensitivity.

Conclusions

- Different kinds of polar molecules could be separated on HILIC columns with good symmetry and efficiency.
- Calibration curve of sucrose show high loading capacity with high degree of linearity within the experimental range.
- System suitability studies (sucrose) show that the analyses could be reproduced with very low %RSD in peak parameters using the TSKgel NH_2-100 column.
- The concentration of acetonitrile has considerable effect on the peak parameters such as retention, peak symmetry and efficiency as seen in the analysis of mannitol using a TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm column.
- This study shows that TSKgel NH_2-100 columns are chemically stable.
- Limit of detection of glucose in the ppb level show high sensitivity of this column.
- Melamine and cyanuric acid could be separated simultaneously by HILIC MS/MS using a 3µm TSKgel Amide-80 column.
- 2-AB-labeled glycans released from ZP domain Construct of Murine TGFR3 could be analyzed by a 3µm TSKgel Amide-80 Column; isobaric glycoforms could be identified by MS/MS.
- Biogenic amines in tuna could be monitored using a TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm column from the samples frozen at -20°C and room temperature.
- Overall, this study shows that TSKgel NH_2-100 and TSKgel Amide-80 columns are suitable for the analysis of different kind of polar molecules.

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

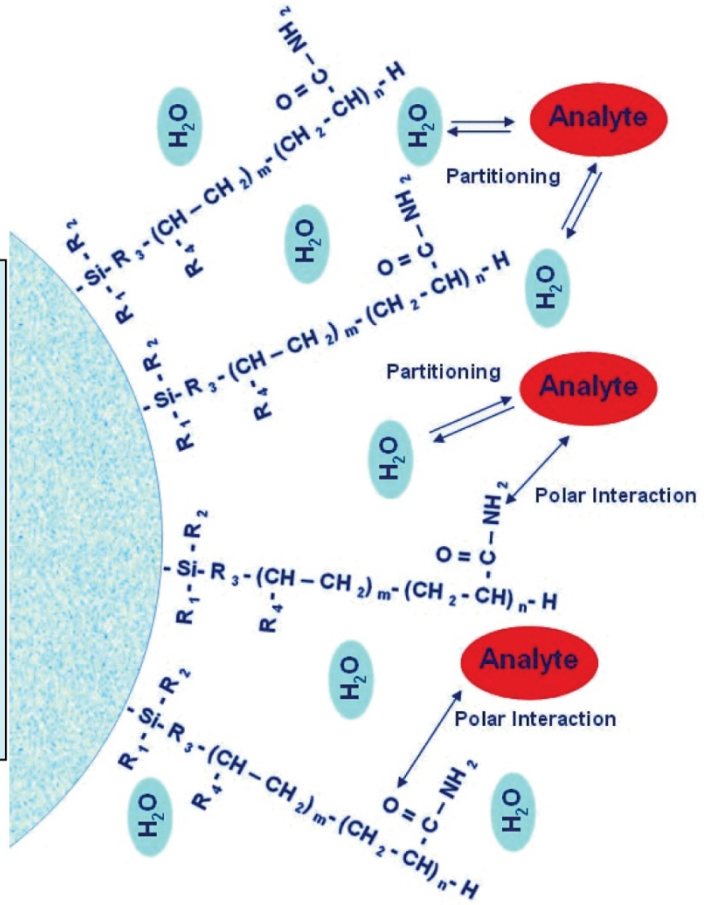
- Saccharides are fundamental substances that express various bioactivities and may exist independently or form complexes with proteins or lipids.
- Saccharides can be classified into monosaccharides, disaccharides, oligosaccharides, polysaccharides etc., based upon the degrees of polymerization and 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.

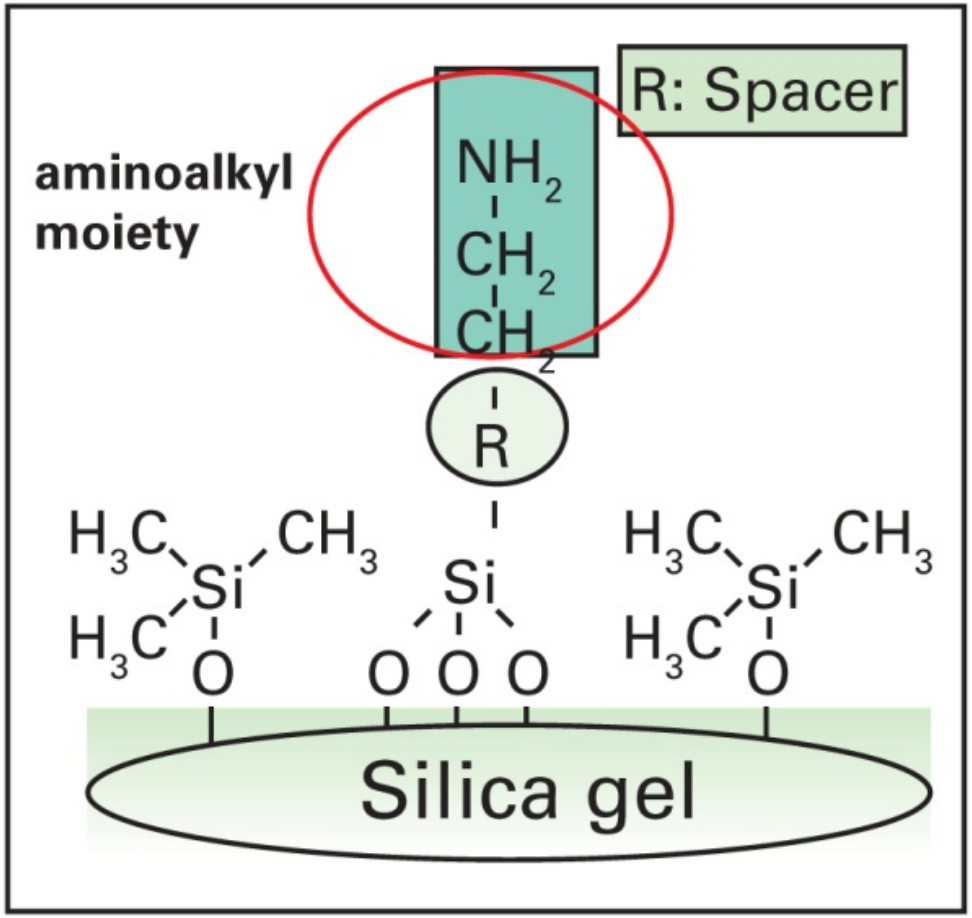
Structure and Mechanism

- 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.

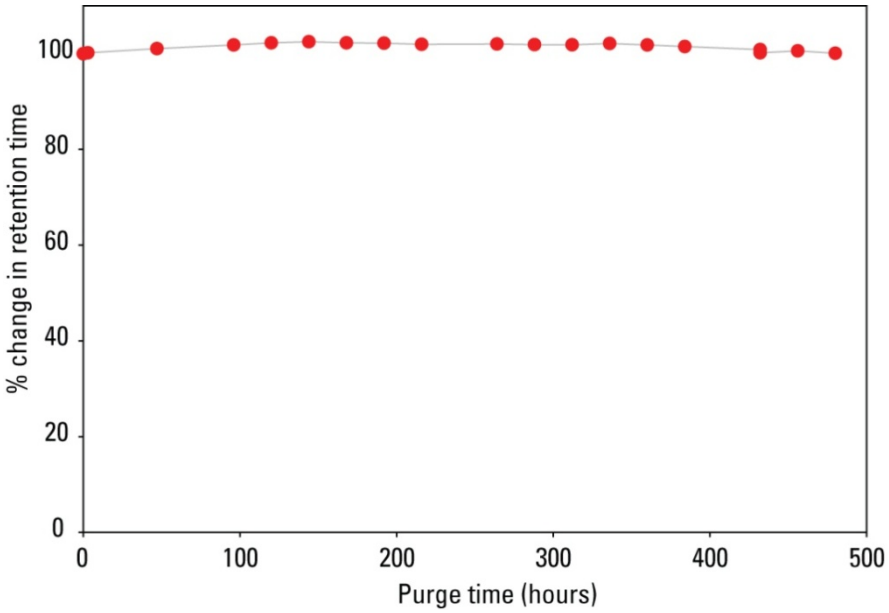
Materials and Methods

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

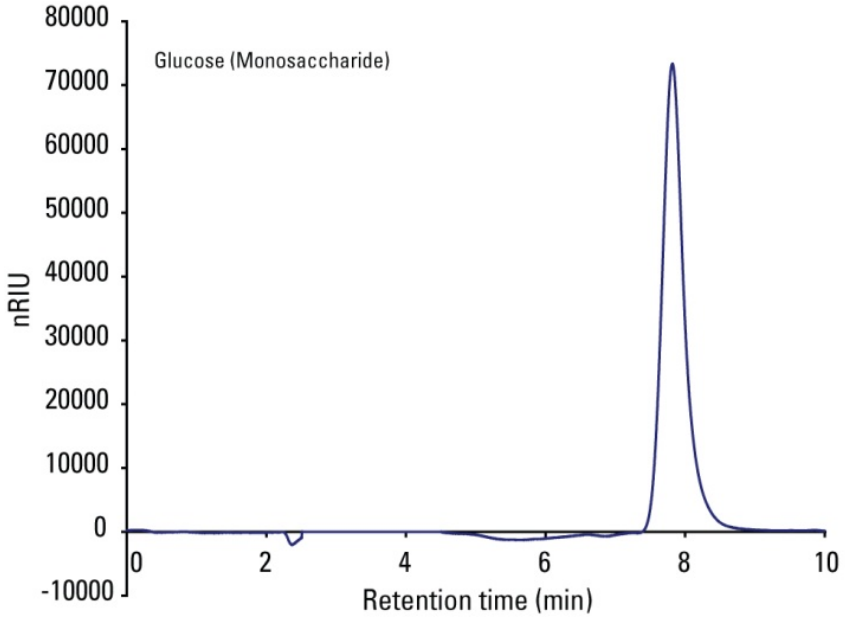
TSKgel NH_2-100 Column Stability



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

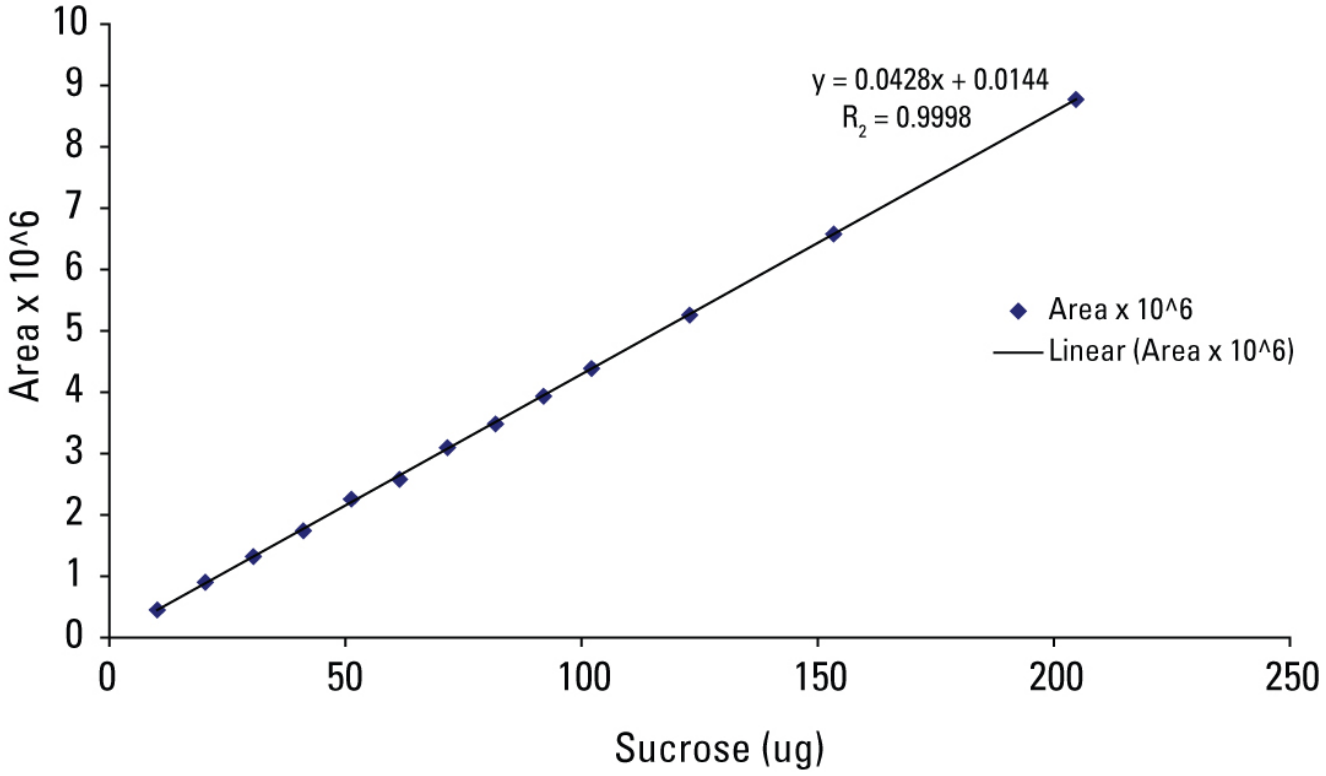
System Suitability Study

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

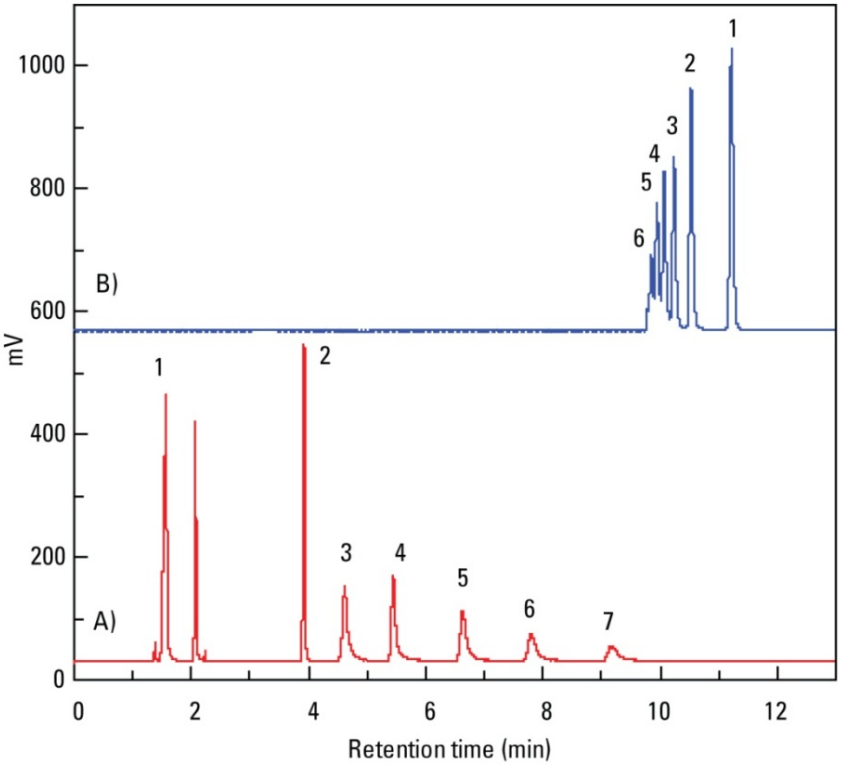
Three consecutive injections of sucrose yielded a very consistent results for all peak parameters that determine the suitability of the system and method.

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

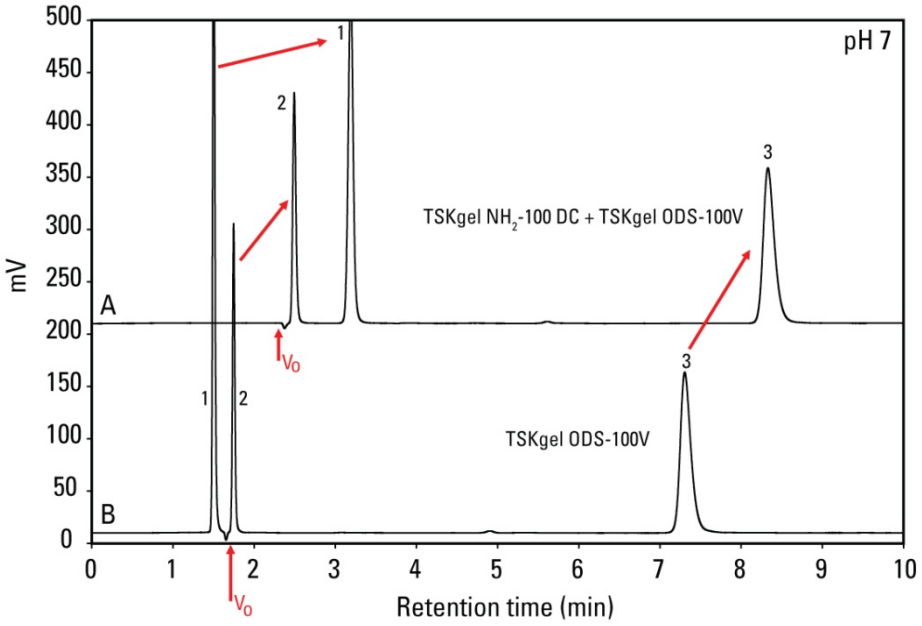


Columns:	A) TSKgel NH_2-100, 3µm, 2.0mm ID x 15cm
	B) TSKgel ODS-100V, 3µm, 2.0mm ID x 15cm
Mobile phase:	A: A) H_2O/ACN (10/90) + 0.1% TFA
	B) H_2O + 0.1% TFA
	B: A) H_2O/ACN (10/90) + 0.1% TFA
	B) ACN + 0.1% TFA
Gradient:	0% B (0min), 40% B (15min), 0% B (17min)
Flow rate:	0.20mL/min
Detection:	UV@313nm
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

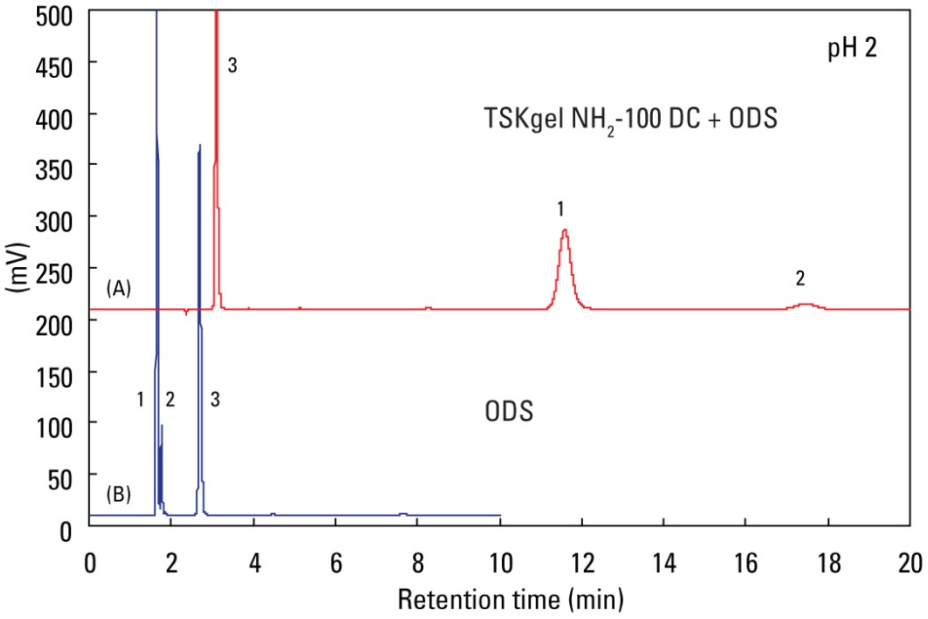


Columns:	A: TSKgel NH_2-100 DC, 3µm, 4.6mm ID x 5cm + TSKgel ODS-100V, 3µm, 4.6mm ID x 15cm B: TSKgel ODS-100V, 3µm, 4.6mm ID x 15cm
Mobile phase:	50mmol/L phosphate buffer, pH 7.0/MeOH = 30/70
Flow rate:	1.0mL/min
Inj. volume:	5µL
Temperature:	40°C
Detection:	UV@210nm
Samples:	1. maleic acid (50mg/L)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

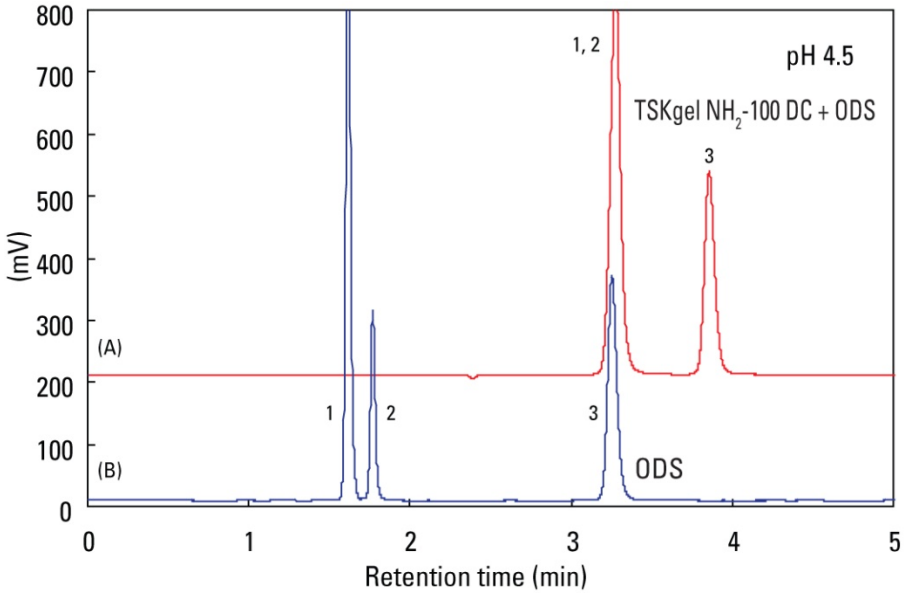


Column:	A) TSKgel NH_2-100 DC, 3µm, 4.6mm ID x 5cm + TSKgel ODS-100V, 3µm, 4.6mm ID x 15cm (B) TSKgel ODS-100V, 3µm, 4.6mm ID x 15cm
Mobile phase:	H_2O/MeOH/H_3PO_4 = 30/70/0.1, pH 2
Flow rate:	1.0mL/min
Detection:	UV@210 nm
Temperature:	40°C
Injection vol.:	5µL
Samples:	1. maleic acid (50mg/L) 2. p-toluene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

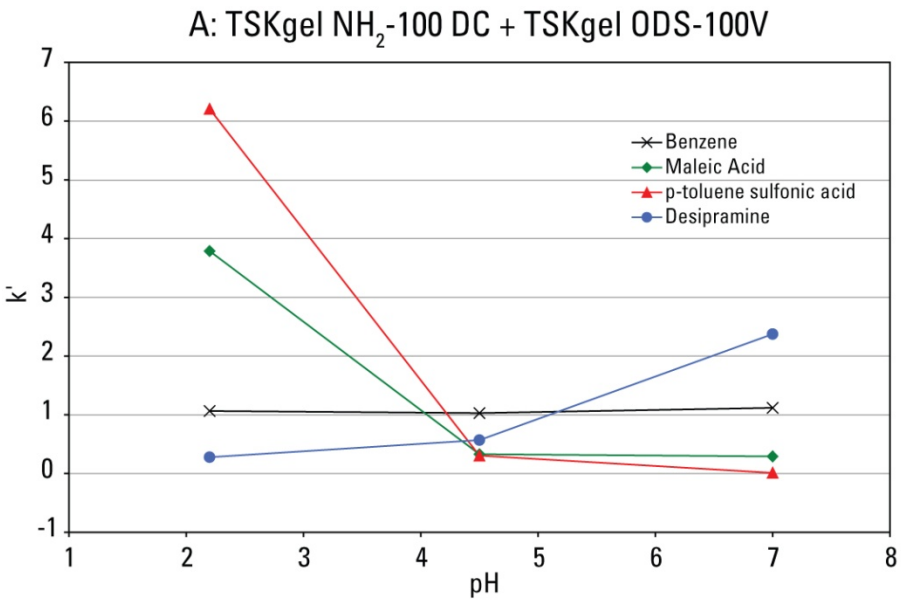
- At pH 4.5, both maleic acid and p-toluene sulfonic acid show substantial retention on the TSKgel NH2-100 DC, 3µm column but elute at the same retention time, while desipramine is retained longer.

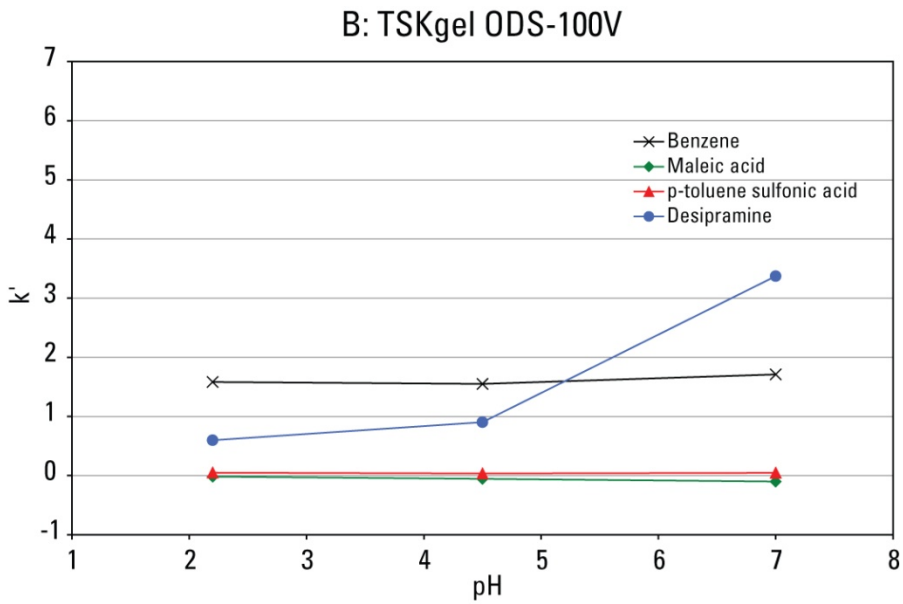
Analysis of a Basic Drug and Organic Acids by Coupling a HILIC and a Reversed Phase Column: (3) pH 4.5



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





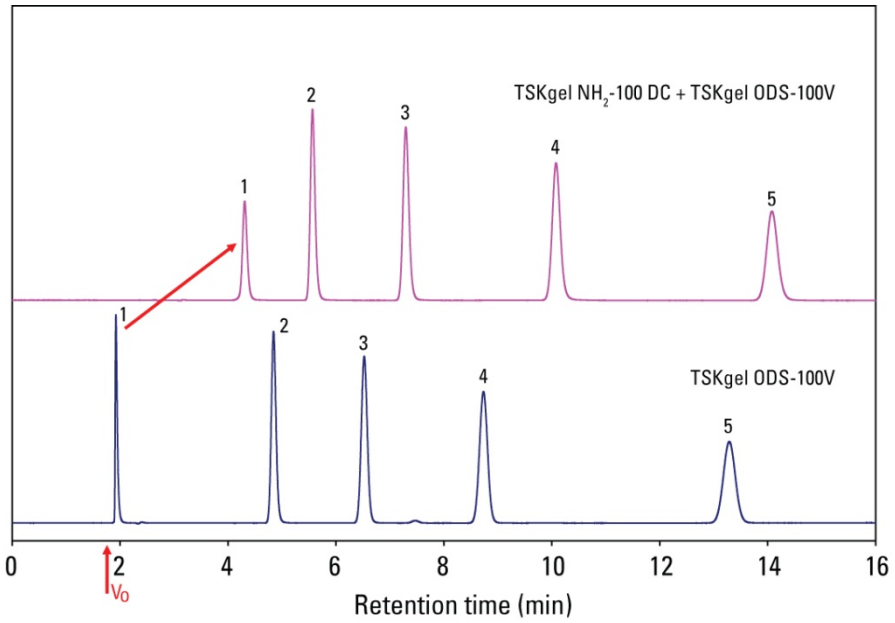
Column:	A: TSKgel NH_2-100 DC, 3µm, 4.6mm ID x 5 cm + TSKgel ODS-100V, 3µm, 4.6mm ID x 15 cm B: TSKgel ODS-100V, 3µm, 4.6mm ID x 15 cm
Mobile phase:	H_2O/MeOH/H_3PO_4 = 30/70/0.1 50mmol/L NaH_2PO_4, pH 4.5/MeOH = 30/70 50mmol/L phosphate buffer, pH 7.0/MeOH = 30/70

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.