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

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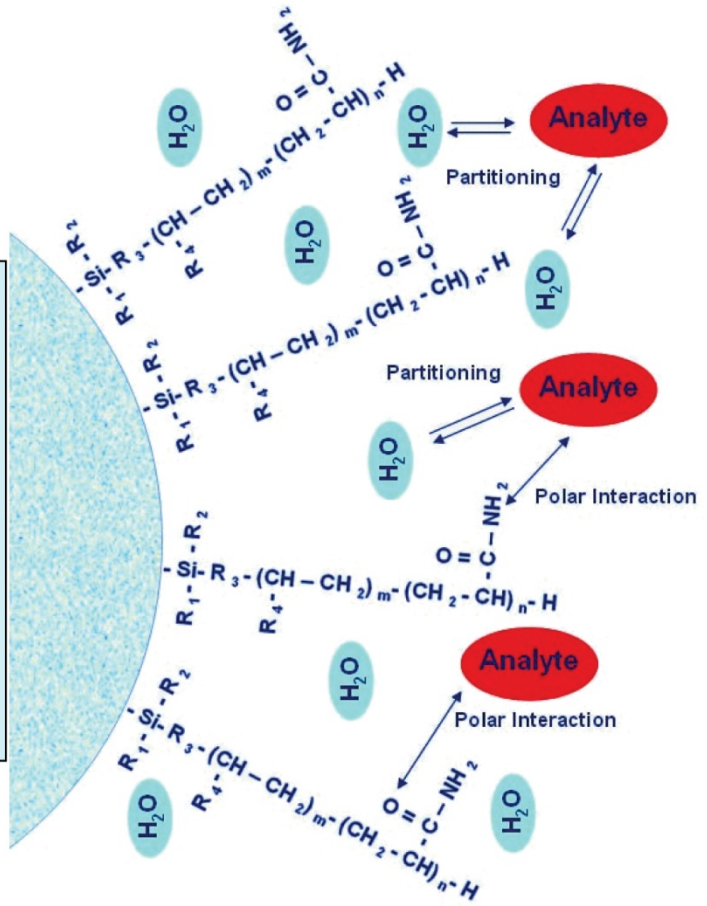
Introduction

- 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

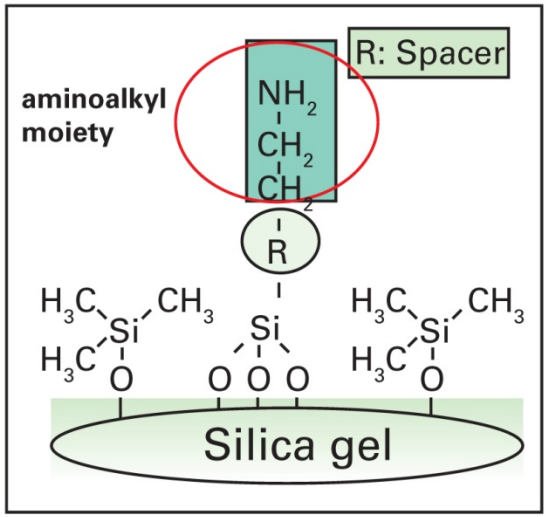
- Polar stationary phase as in normal phase LC
- Mobile phase similar to reversed phase (high organic)
- Elution in order of increasing hydrophylicity



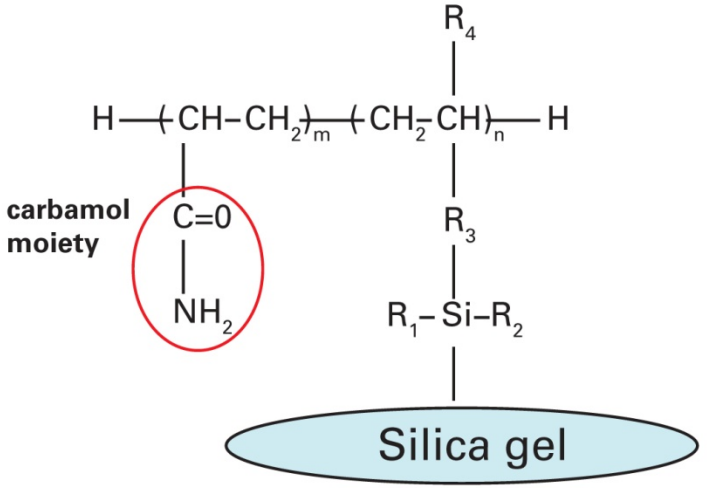
Mechanism of Hydrophilic Interaction Liquid Chromatography (HILIC)

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**

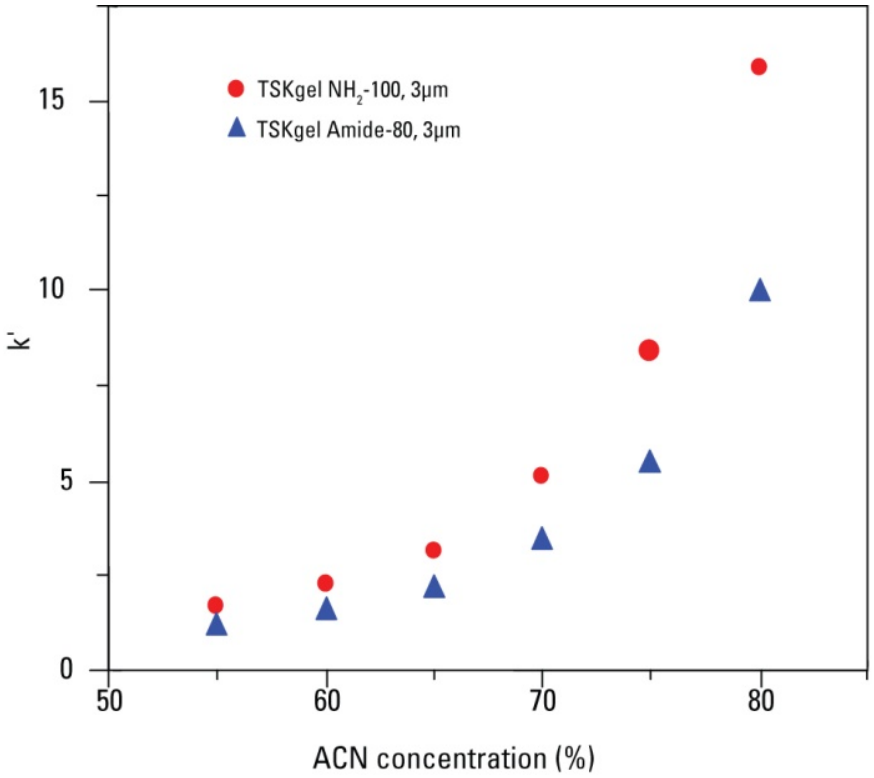
TSKgel Amide-80	
Particle size (µm)	3
Pore size (nm)	10
Surface area (m^2/g)	450
Functionality	Carbamoyl group

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.

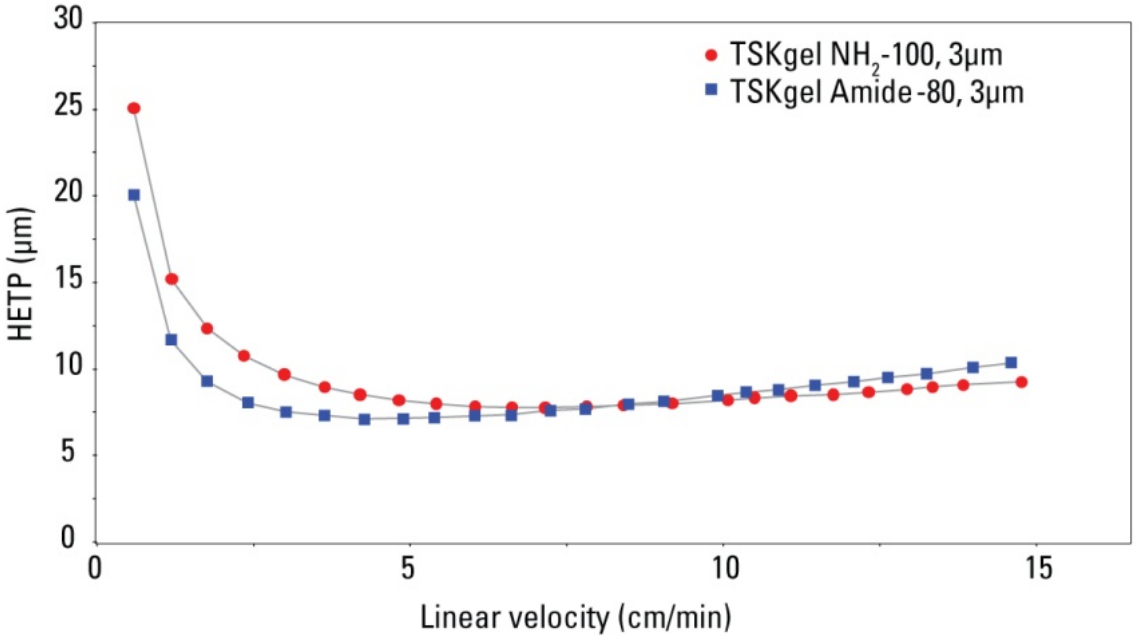
Retention of TSKgel HILIC Columns



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

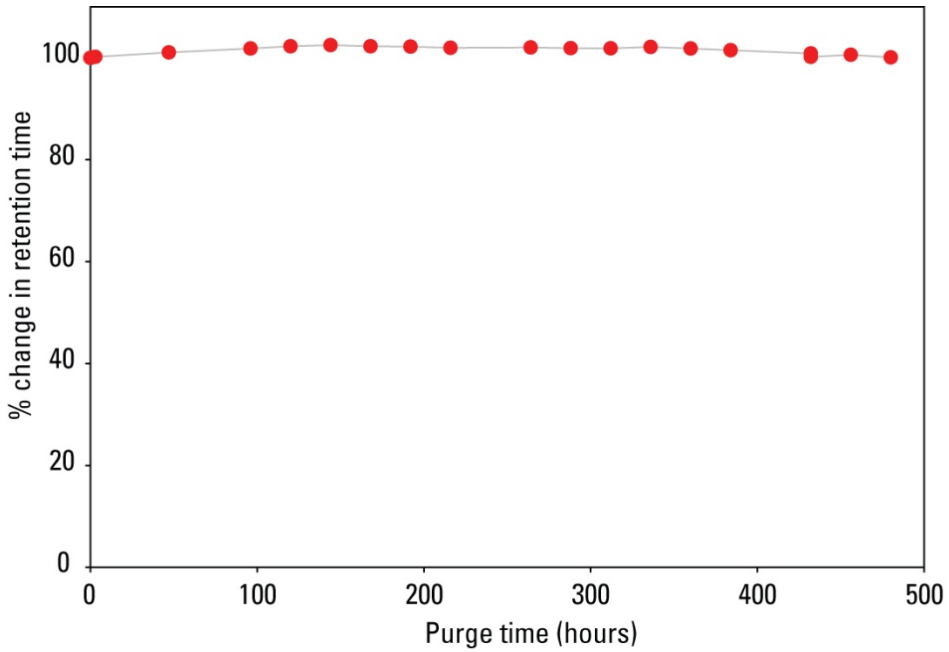


TSKgel NH_2-100, 3µm, 4.6mm ID x 15cm
TSKgel Amide-80, 3µm, 4.6mm ID x 15cm

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_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 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

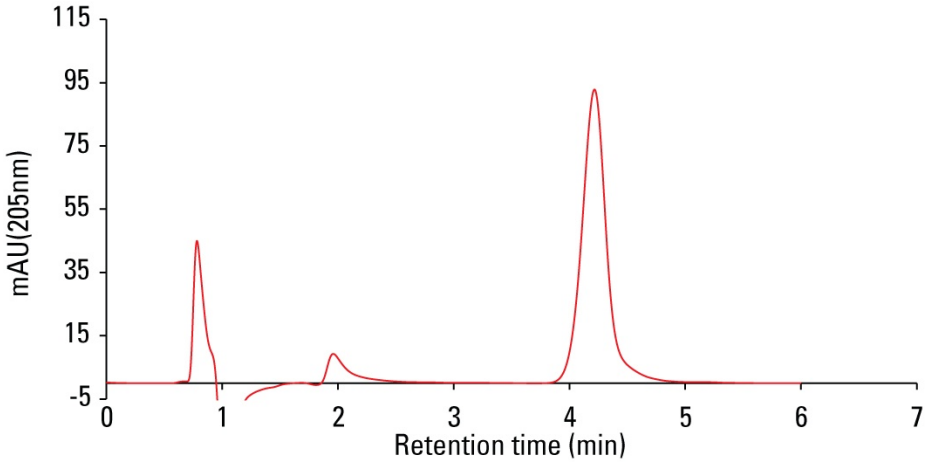
Material and Methods (contd.)

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.

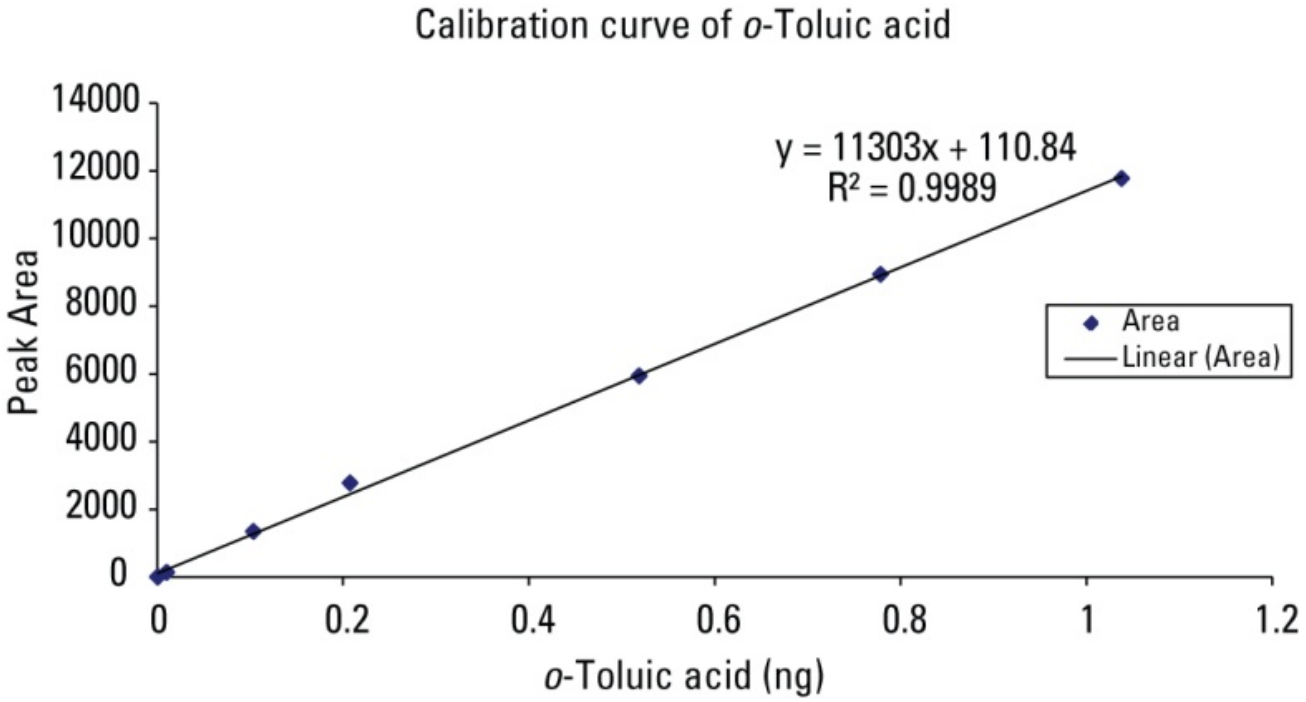
High purity chemicals and HPLC grade solvents were used for the preparation of stock standards, samples and mobile phases.

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



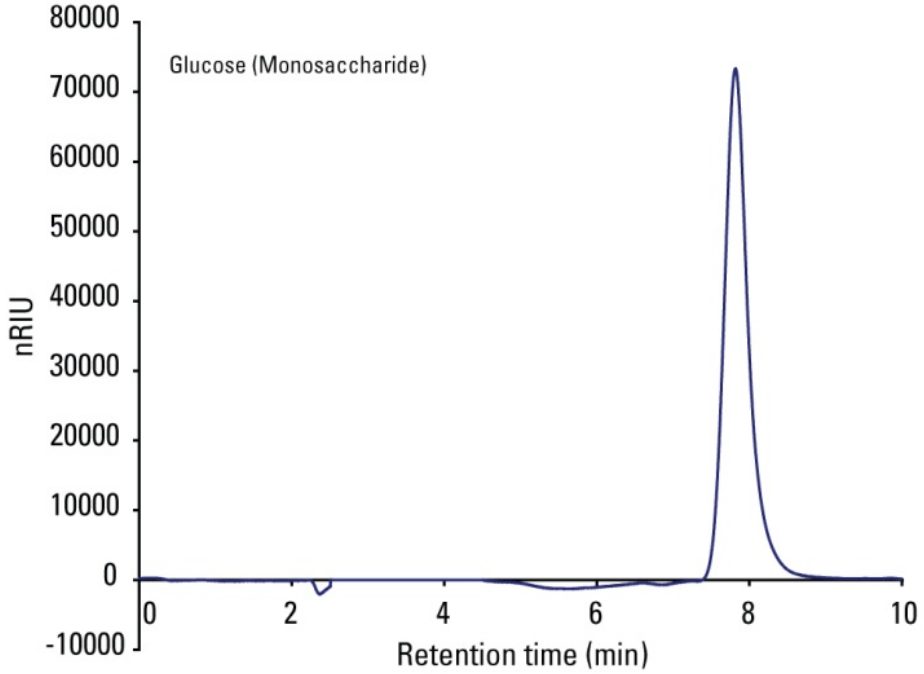
Column:	TSKgel NH_2-100, 3um, 2.0mm ID x 5cm
Mobile phase:	70% ACN:30% 5mmol/L in ammonium acetate in H_2O, pH 4.1
Flow rate:	0.2mL/min
Detection:	UV@210nm
Temperature:	40°C
Injection vol.:	5µL
Sample:	*o*-toluic acid

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

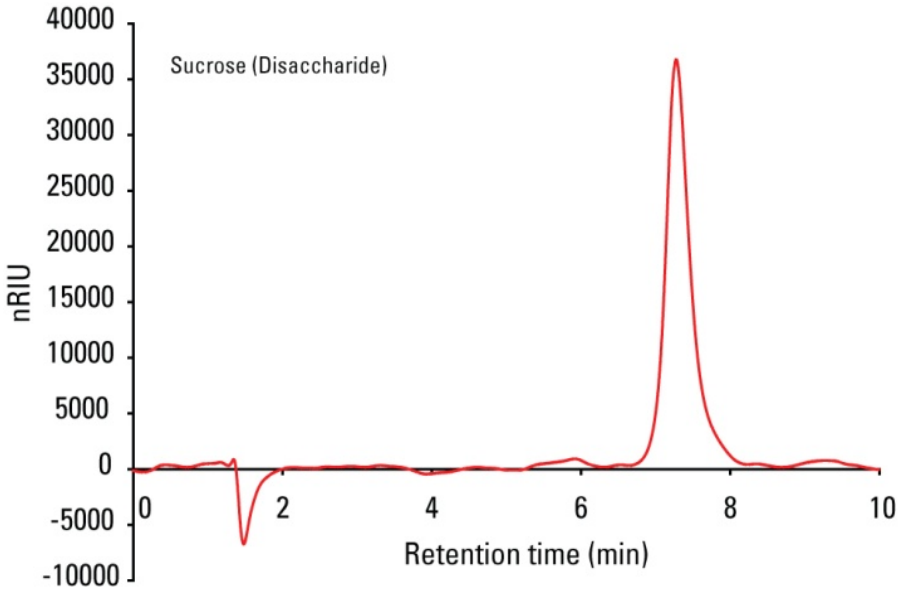


Columns:	TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm
Mobile phase:	80% ACN in H_2O
Flow rate:	0.2mL/min
Detection:	RI
Temperature:	50°C
Injection Vol.:	2µL

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

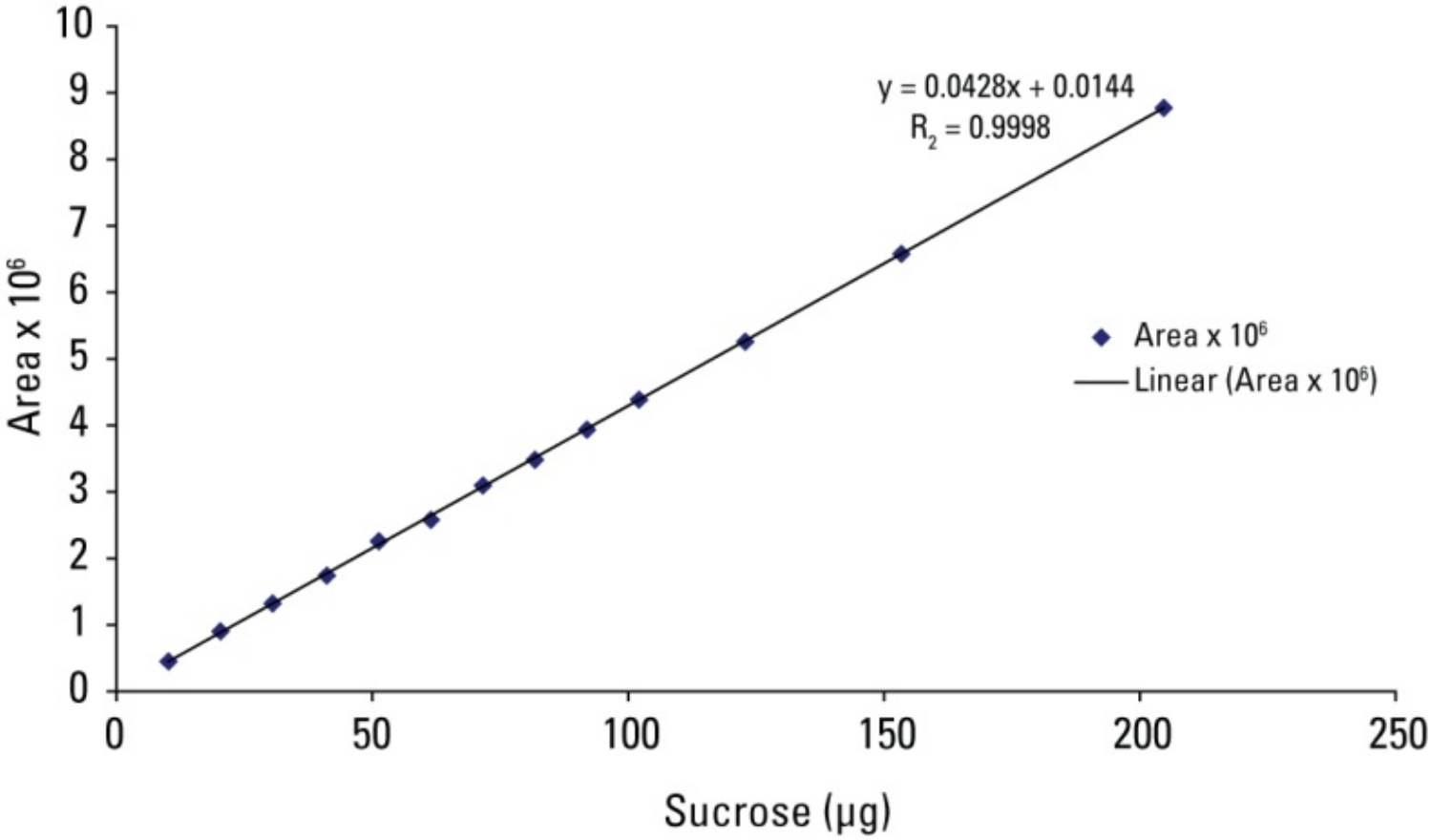
System Suitability Study

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

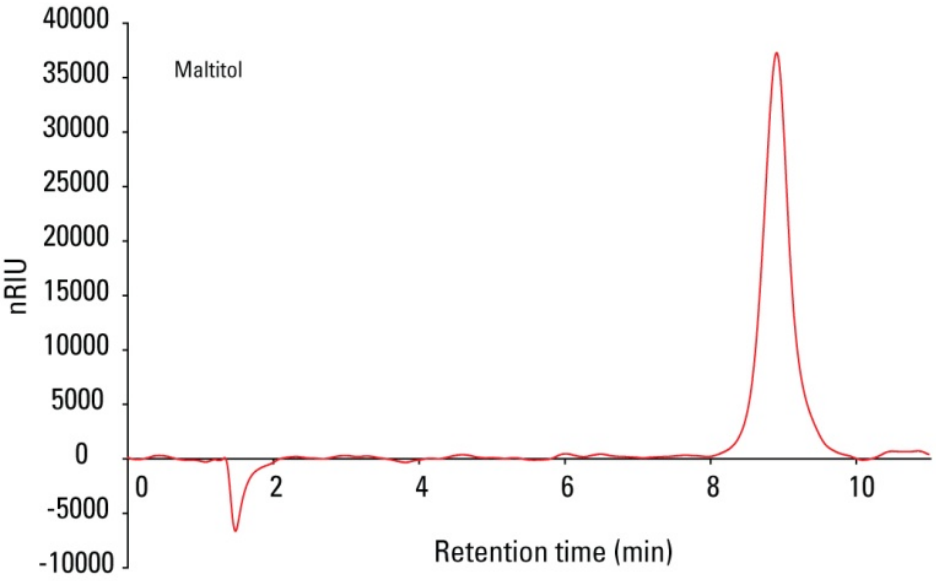
Three consecutive injections of sucrose yielded very consistent results for all peak parameters that determine the suitability of the system and method.

Loading Capacity



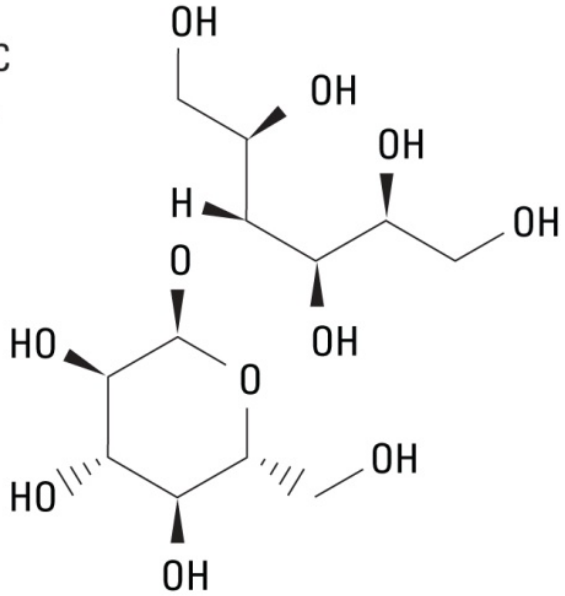
Sucrose can be analyzed with a high degree of linearity over the experimental concentration range shown in this figure.

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

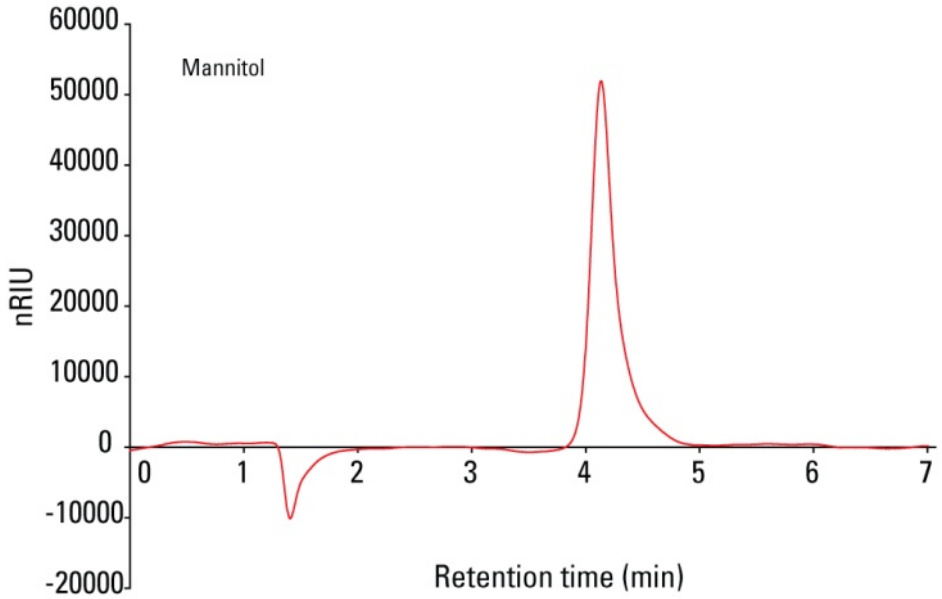


RT	k	Area x 10^6	A_s	Plates
8.908	13.18	104	1.05	3019

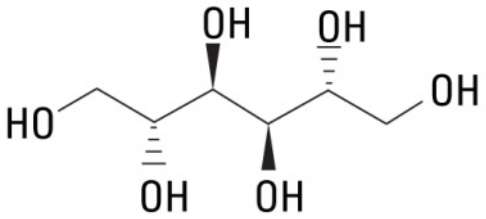
Column:	TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm
Mobile phase:	80% ACN in H_20
Flow rate:	0.2mL/min
Detection:	RI
Temperature:	50°C
Injection vol.:	2µL



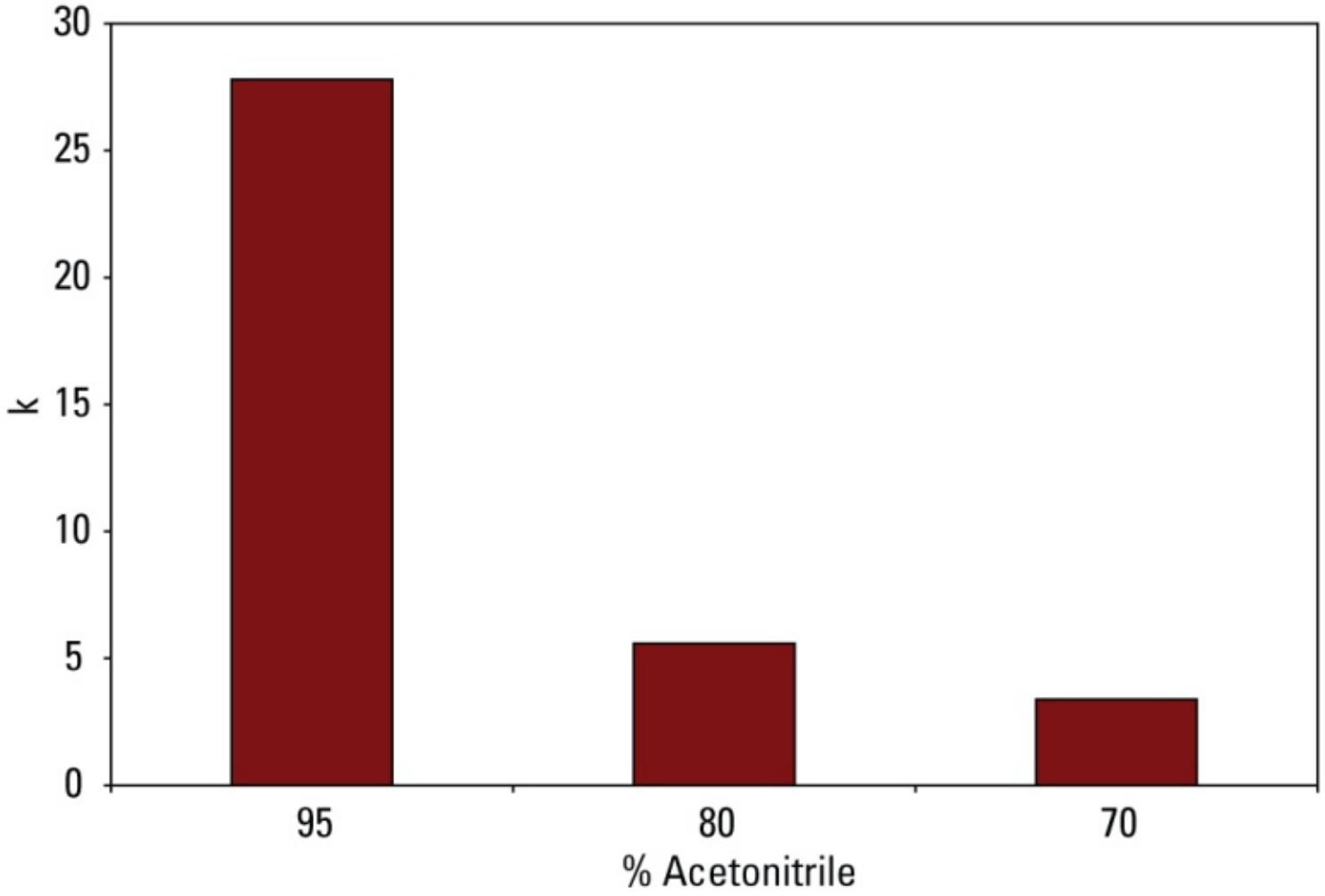
Analysis of Mannitol (polyol or sugar alcohol) using a TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm Column



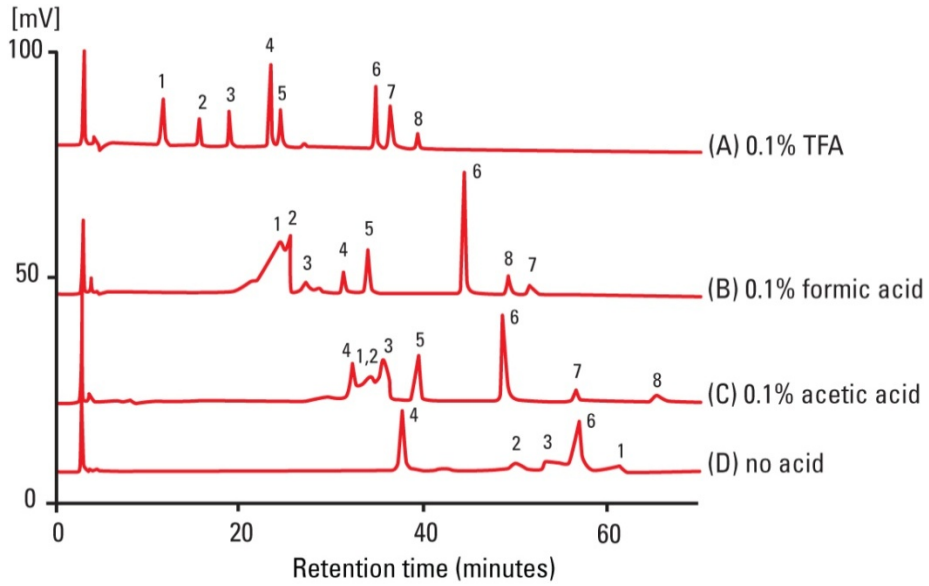
Column:	TSKgel NH_2-100, 3um, 2.0mm ID x 5cm
Mobile phase:	80% ACN in H_20
Flow rate:	0.2mL/min
Detection:	RI
Temperature:	50°C
Injection vol.:	2µL



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

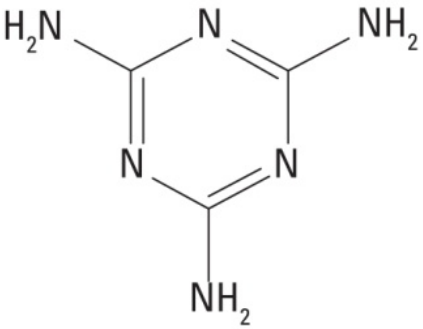


Peak/Peptide Number	Sequence	Recovery from TSKgel Amide-80	(%) Recovery from TSKgel ODS-80T$_S$*
1	FY	96	96
2	FGGF	101	89
3	FLEEI	98	93
4	DYMGWMDP-NH2	90	74
5	NFTYGGF	90	95
6	AGSQ	96	65
7	WAGGDASGE	85	96
8	YGGFMTSQKSQTPLVT	92	96
9	ASTTTNYT	94	89
10	VLSEGEWQLVLHVW AKVEADVAGHGQDI LIRLFKSHPETLEKFD RFKHLKTEAEM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

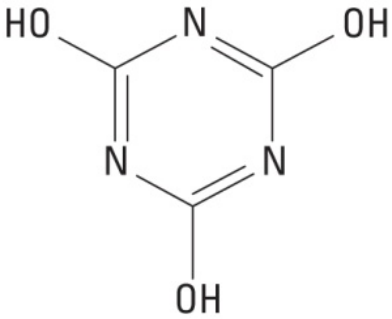
*TSKgel ODS-80T$_S$ run was at 83.3 min. linear gradient of ACN from 5 to 55% in 0.1% TFA

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

↓

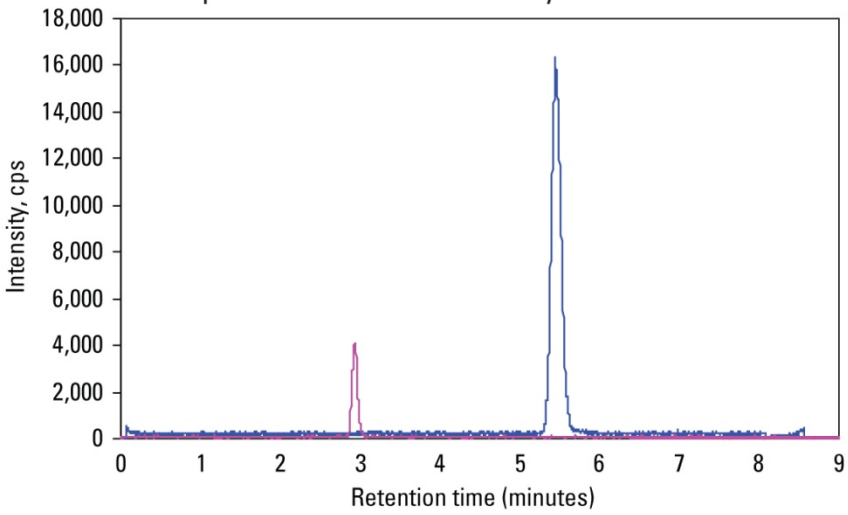
Ultracentrifugation @ 5,000rpm for 5minutes

↓

Filtration (pore size: 0.5µm)

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

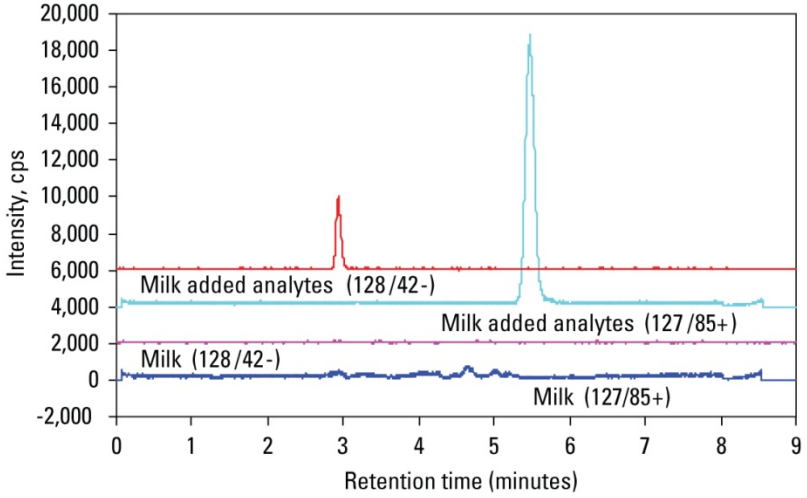
Separation of melamine and cyanuric acid in milk



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



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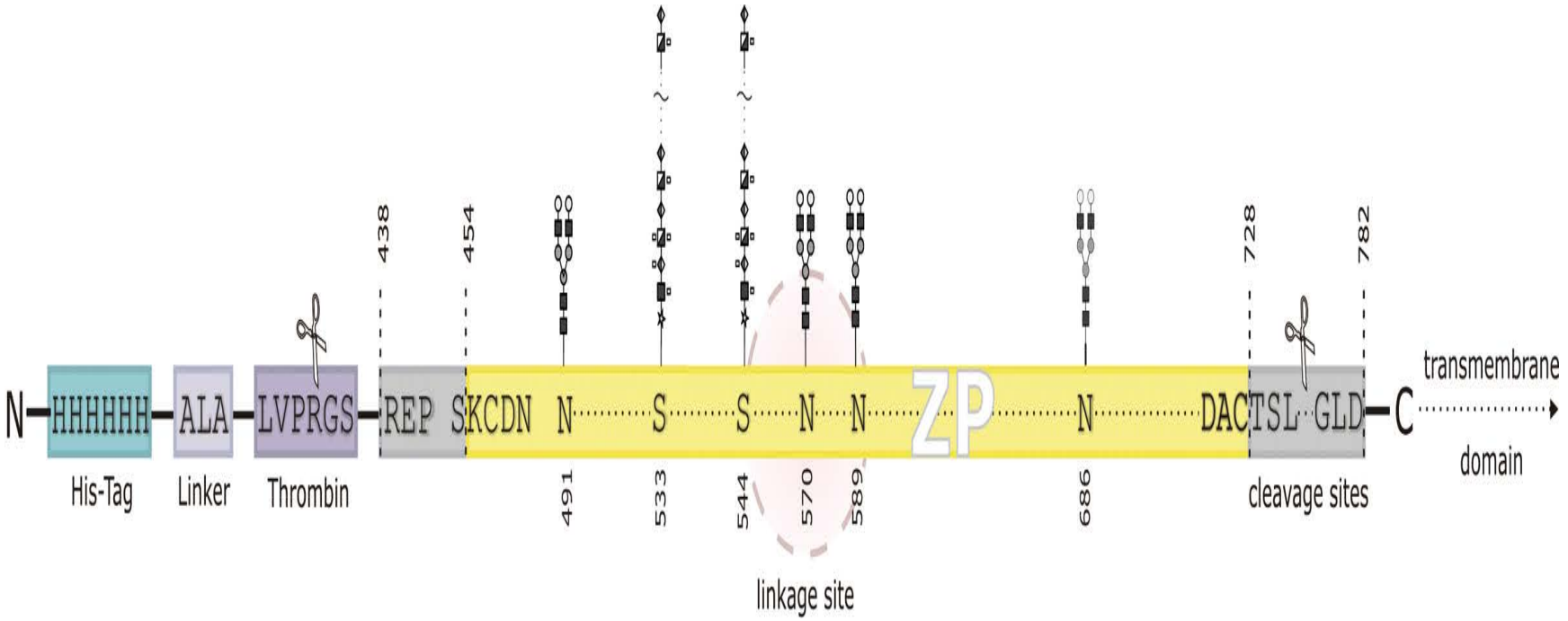
Temperature:	40°C
Injection vol.:	5µL
Instrument:	QTRAP (AB Sciex)
Ion source:	ESI
	127/85+ (melamine)
	128/42- (cyanuric acid)

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

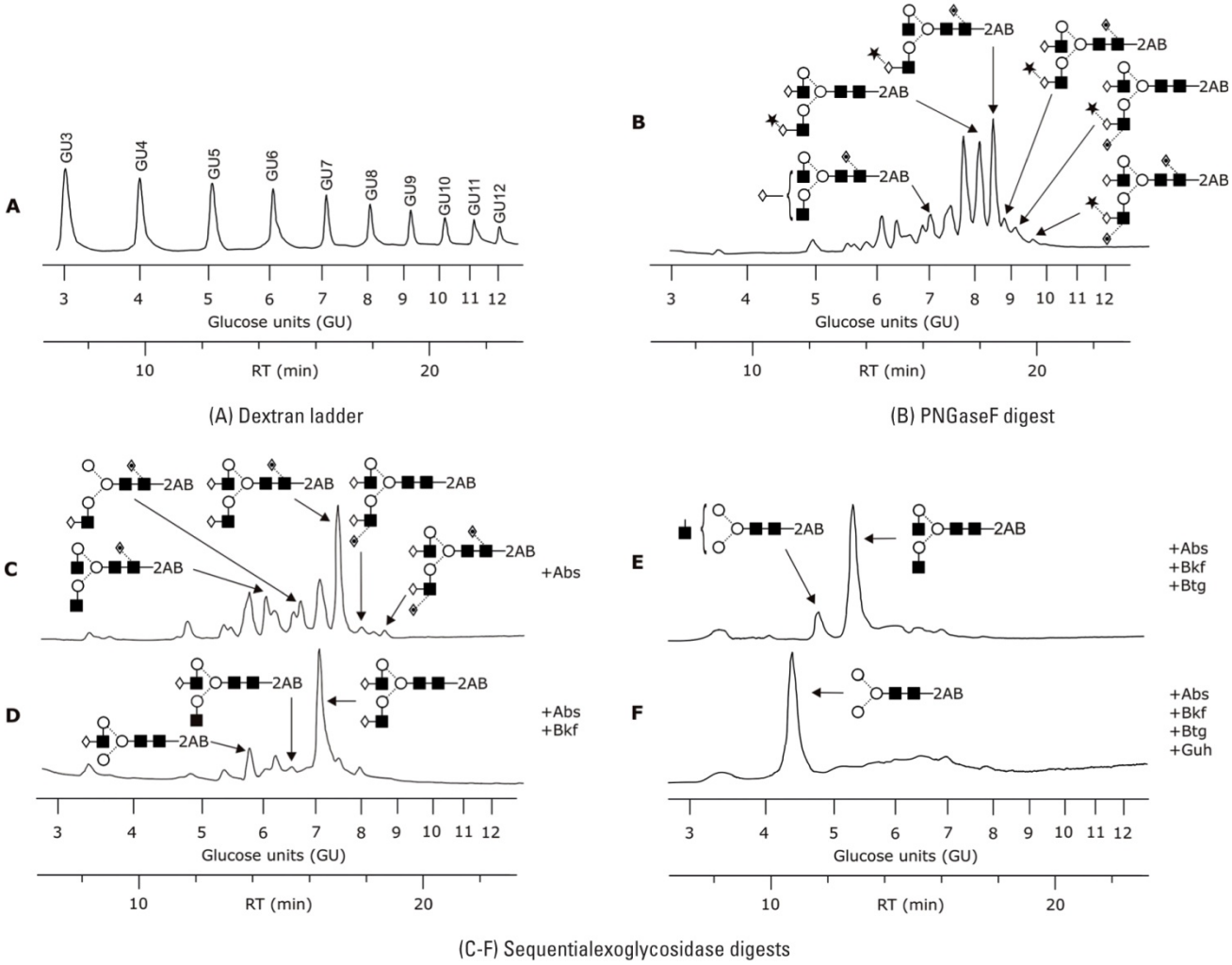
Fluorescence chromatograms of HILIC separations of 2-AB labeled N-glycans released from the recombinant ZP domain construct of murine TGFR-3, were compared to the dextran ladder.

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.

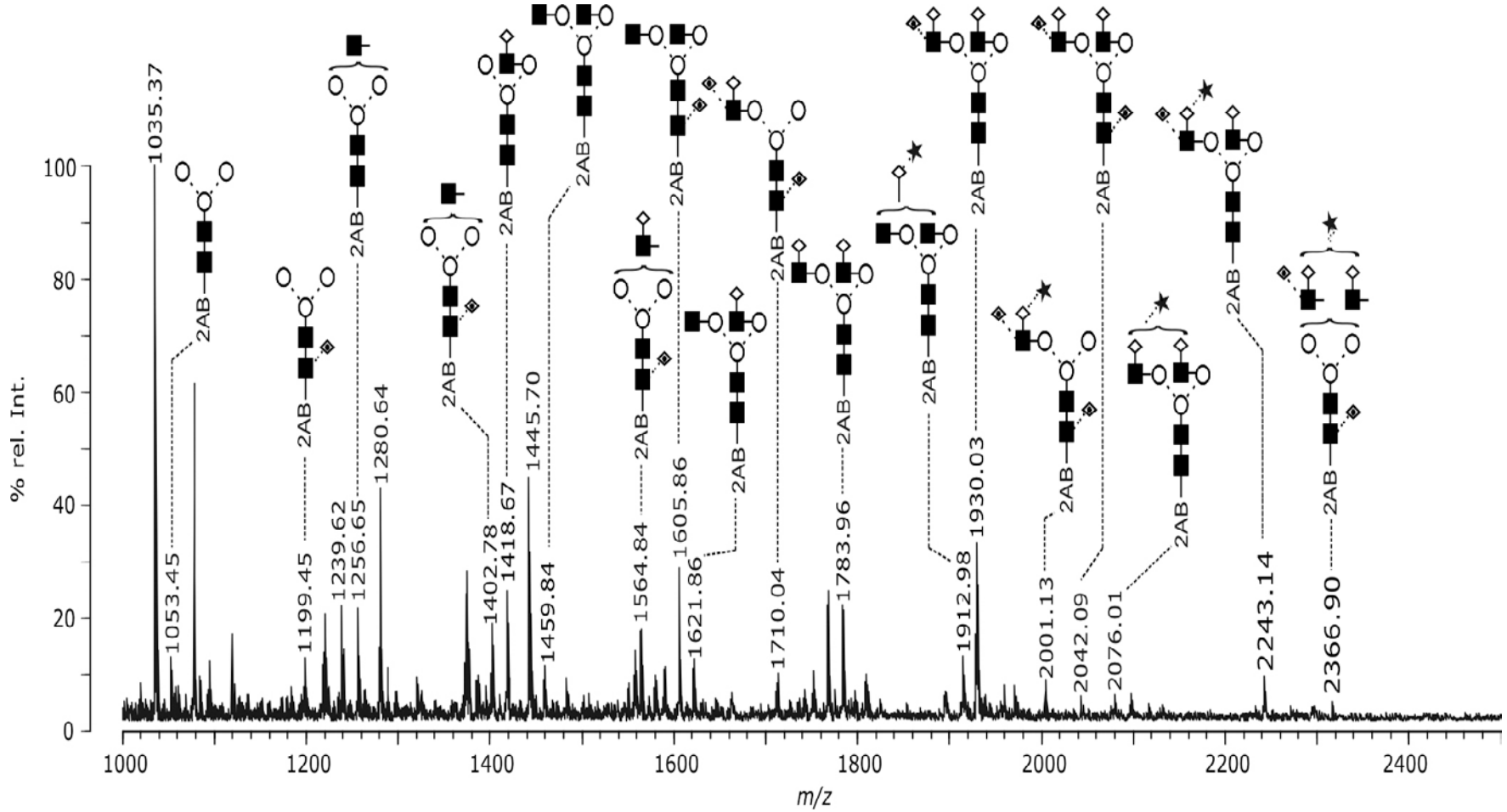
Separations of 2-AB Labeled N-glycans



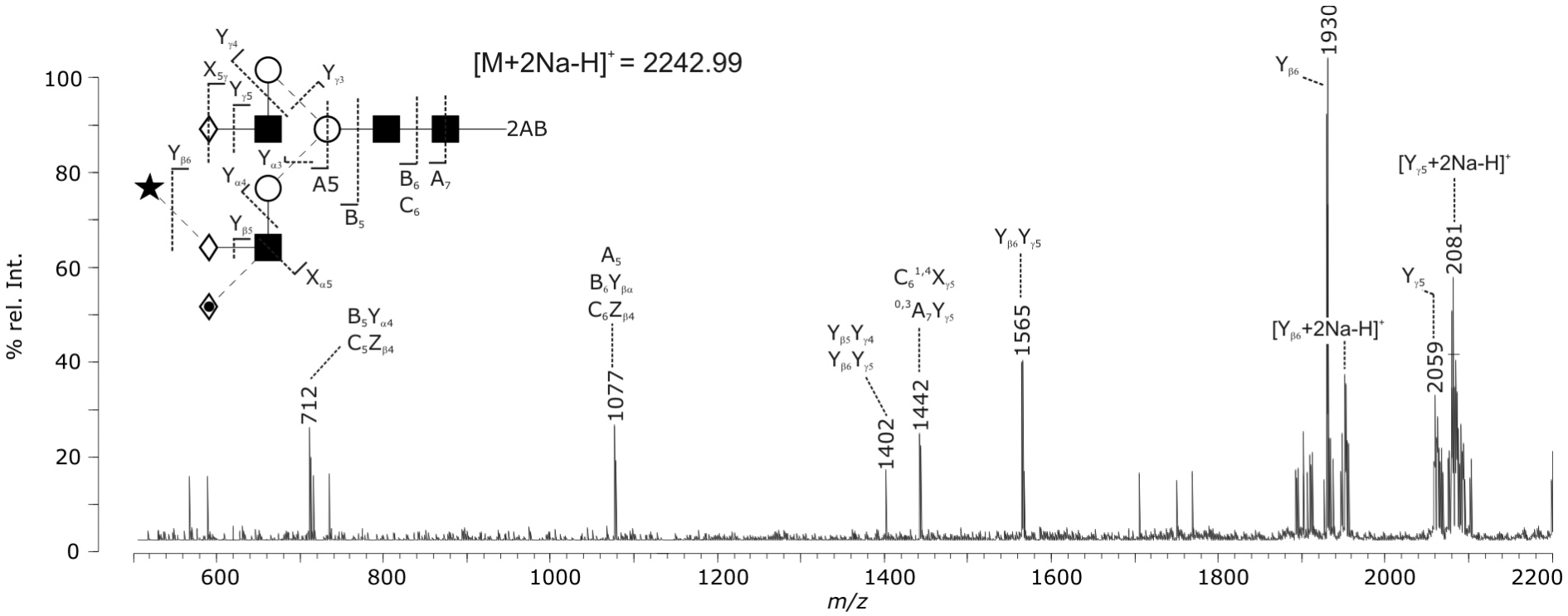
Used exoglycosidase:

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

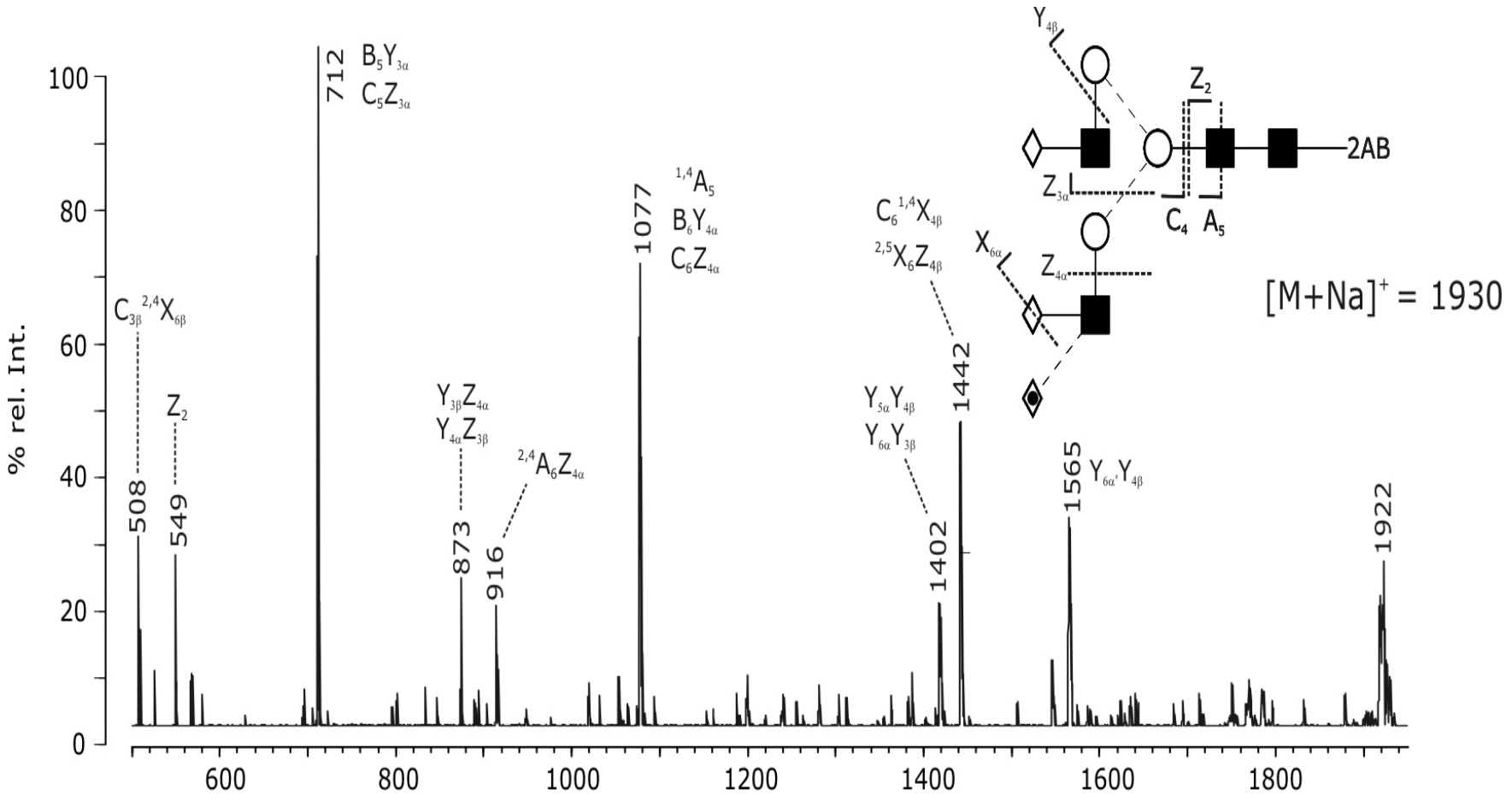
MALDI Mass Spectrum of 2-AB-labeled Glycans Released from ZP domain Construct of Murine TGFR3



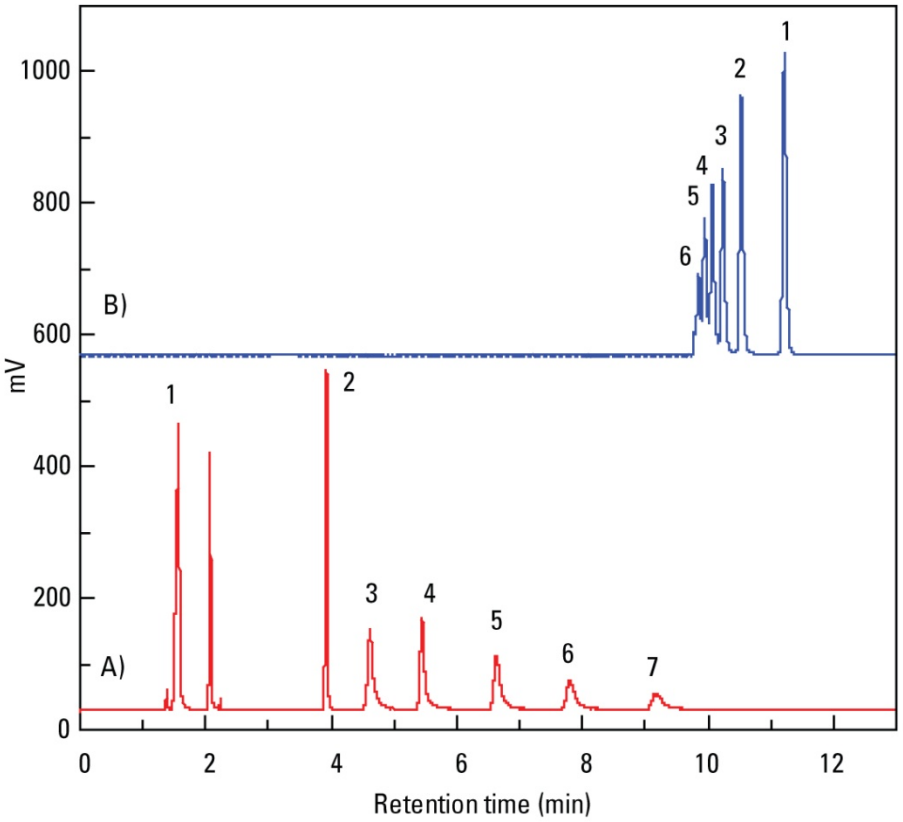
MS2 (CID) Mass Spectrum of m/z 2243



MS3 (CID) Mass Spectrum of m/z 1930



Comparison of Chromatograms of MTX and its Derivatives



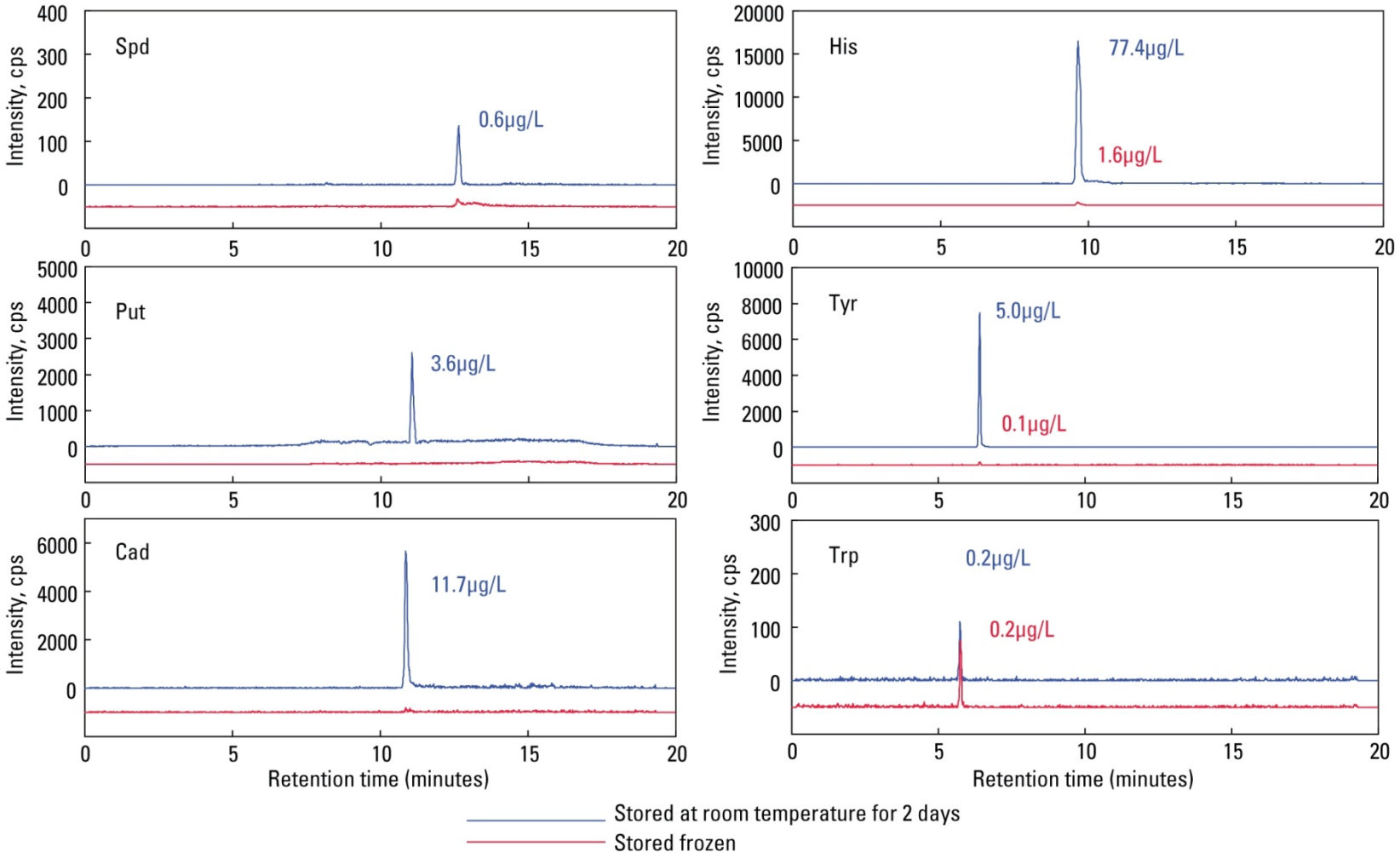
Columns:	A) TSKgel NH_2-100, 3µm, 2.0mm ID x 15cm
	B) TSKgel ODS-100V, 3µm, 2.0mm ID x 15cm
Mobile phase:	A: A) H_2O/ACN (10/90) + 0.1% TFA
	B) H_2O + 0.1% TFA
	B: A) H_2O/ACN (10/90) + 0.1% TFA
	B) ACN + 0.1% TFA
Gradient:	0% B (0min), 40% B (15min), 0% B (17min)
Flow rate:	0.20mL/min
Detection:	UV@313nm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

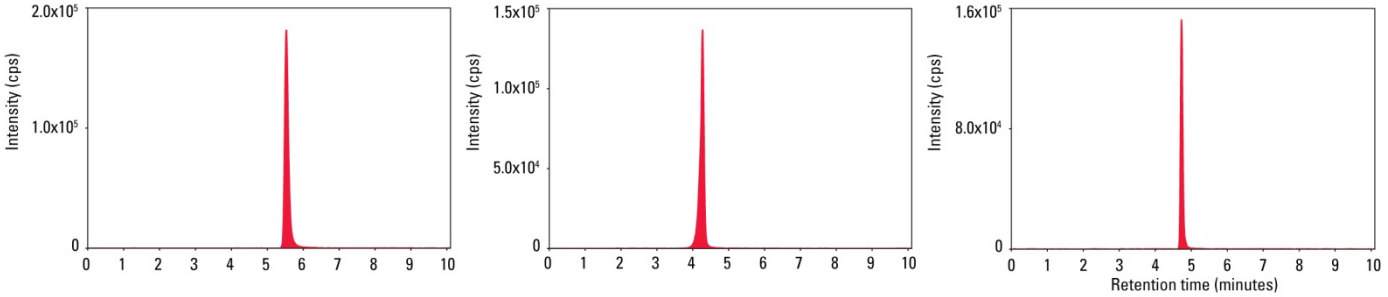
Analytical Conditions of LC/MS/MS

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

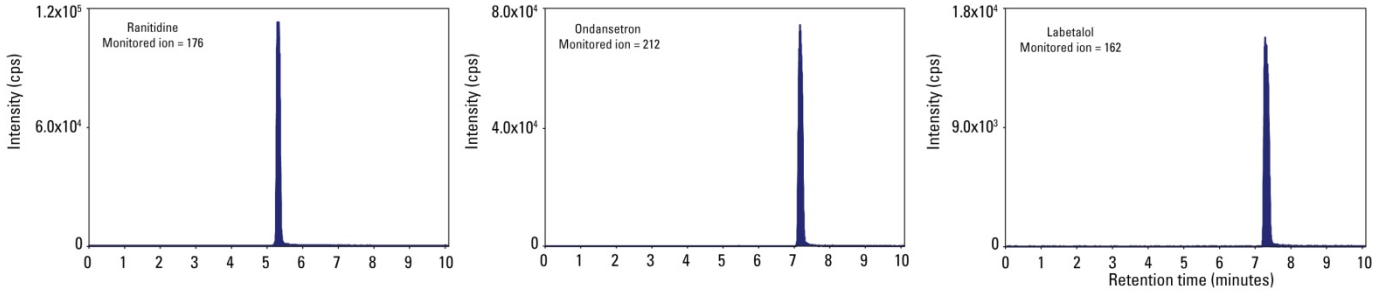


LC/MS/MS Analysis of Polar Basic Drugs - HILIC or RPC Mode?



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.