GLYCO-PAK N COLUMNS

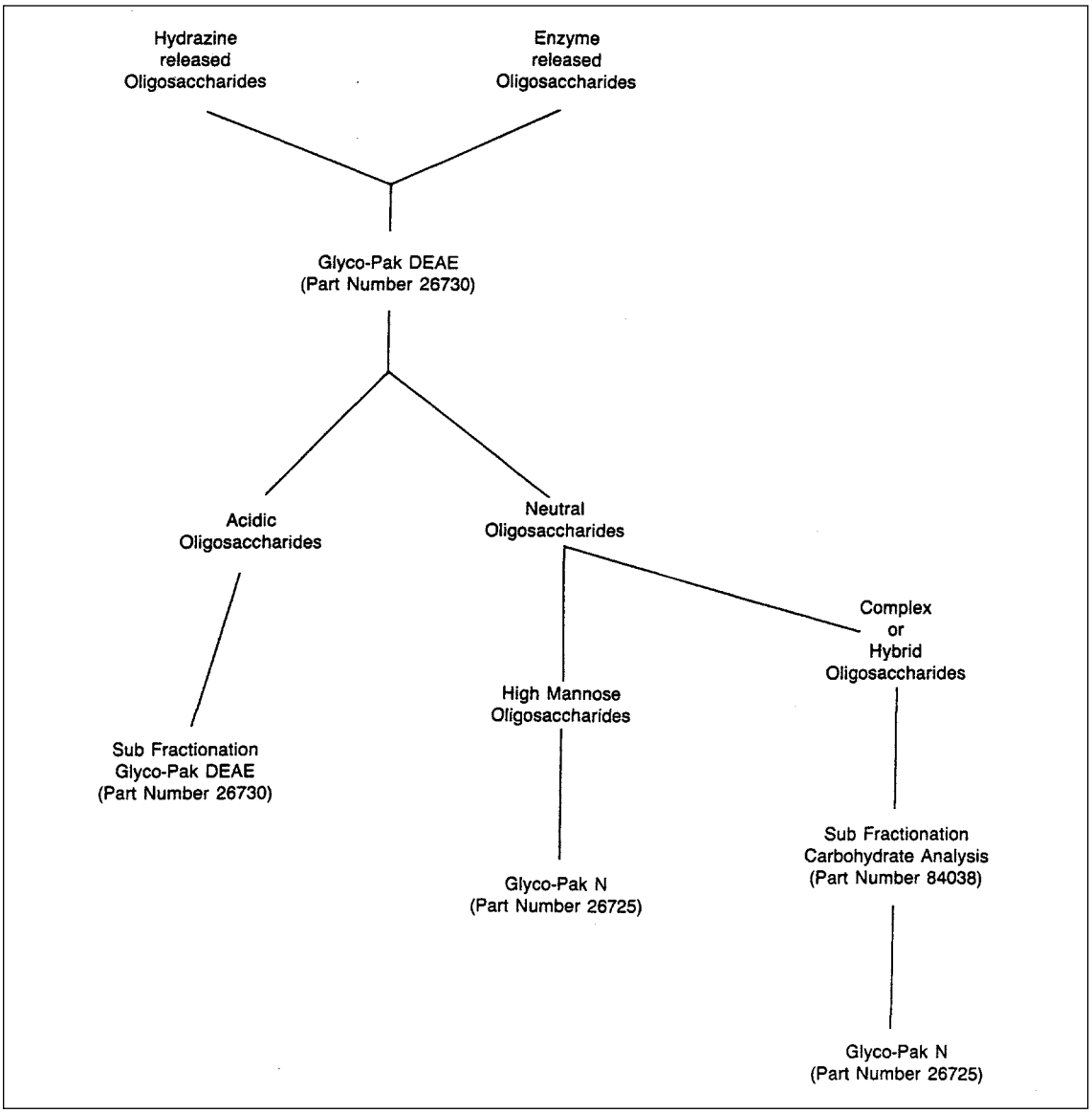
I. INTRODUCTION

Waters Glyco-Pak™ N column is a polymeric column designed for the fractionation of neutral oligosaccharides released from glycoconjugates.

CAUTION: Improper equilibration of this column may result in decreased column performance. Please read Section III of this manual before attempting to use this column.

CONTENTS

Figure 1: Typical Isolation Scheme



Oligosaccharides released by hydrazinolysis or enzyme treatment may be simultaneously separated into neutral and multiple acidic fractions (depending on the number of sialic acids or degree of sulfation) on the Glyco-Pak DEAE column (P/N 26730). Acidic fractions may be further purified on the Glyco-Pak DEAE by changing the mobile phase and/or gradient conditions.

High mannose and simple mixtures of complex or hybrid oligosaccharides can then be fractionated directly on the Glyco-Pak N column (P/N 26725). For more complex mixtures a subfractionation on a different column (Carbohydrate Analysis column and others) may be of value before a final purification on a Glyco-Pak N column. Use these procedures to ensure that you obtain quality results and take full advantage of the features your Waters column offers.

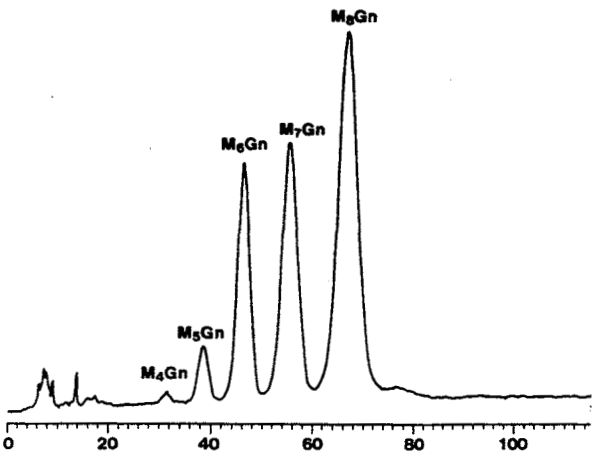
a. Mode of Action

The Glyco-Pak N column operates in a liquid/liquid partition mode. In this mode, retention tunes and resolution are very sensitive to small changes in the composition of the mobile phase. To insure retention time reproducibility, mobile phase should be prepared by weight or in sufficiently large batches to insure consistency.

Conditions

Sample:	Endo H released high mannose oligosaccharides
Mobile Phase:	Acetonitrile/water (70:30)
Flow Rate:	1 ml/min
Detection:	197 nm

Figure 2: Endo H Released High Mannose Oligosaccharides



Conditions

Sample:	Mixed oligosaccharide standards
Mobile Phase:	Acetonitrile/water (d8:32)
Flow rate:	l ml/min
Detection:	200 nwm

Figure 3: Mixed Hydrazine Released Oligosaccharide Standards (See Figure 4 for structures.)

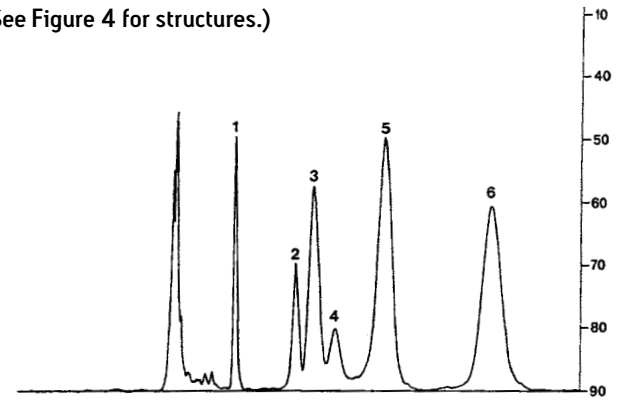
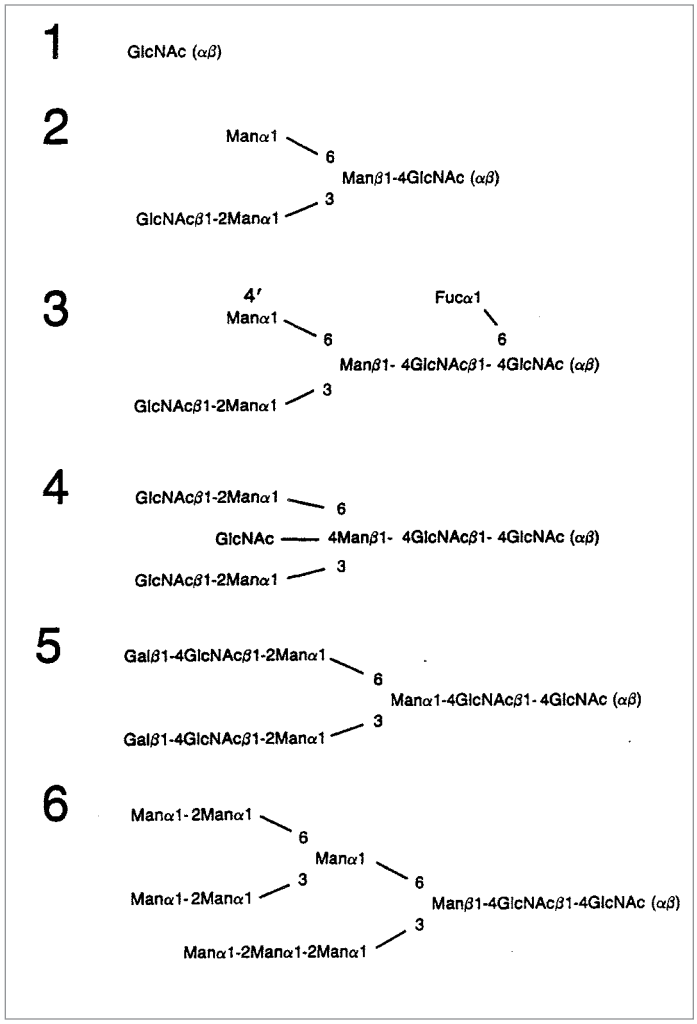


Figure 4: Neutral Oligosaccharide Structures*



*Separation of Reducing Oligosaccharides Derived from Glycoproteins by HPLC on a Polymeric Support, Bendiak, B.; On, J.; Brockhausen, I.; Vella, G.; Phoebe, C.; in press.

II. INSTALLATION

CAUTION: Excessive pressure will damage the Glyco-Pak N Column. Do not exceed a flow rate of 1.0 m1/min or backpressure of 500 psi.

a. Column Installation

Remove the end-cap fittings from your Glyco-Pak N column with a 5/16-inch wrench. Save the end-cap fittings to recap the column when it is removed from the system.

Mobile phase flow direction for the Glyco-Pak N column is indicated by an arrow on the column label.

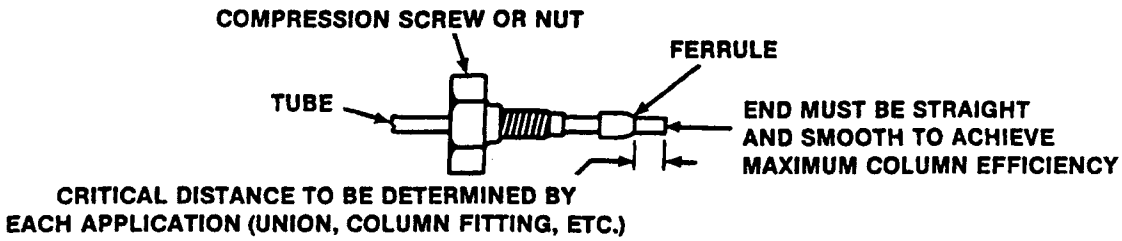
Note: When changing columns, always replace the ferrule. Carefully reseat the ferrule to avoid creating dead volume. Improper seating of the ferule will result in reduced resolution on the Glyco-Pak N column.

Follow the next four steps of this procedure to cut tubing to connect a new steel column, or to improve the end connections on your existing fittings.

1. Using a three-cornered file with a cutting edge, scribe the circumference of the tubing at the desired break.
2. Grasp the tubing on both sides of the scribe mark with cloth-covered pliers (to prevent marring the tube surface) and gently work the tube back and forth until it separates.
3. Slide the compression fitting, followed by the ferrule (large end of the taper first) over the tube. Be certain to bottom the tube in the fitting seat to assure a leak-free connection.
4. Use a 5/16-inch wrench to install the column in the system.

Note: Attach a union in place of the column and flush the lines before installing the column. This flushes previous, possibly incompatible mobile phases from the system.

Figure 5: Ferrule and Compression Assembly



b. Solvent and Sample Preparation

Refer to the following list for mobile phase and sample considerations:

- Use LC grade solvents filtered through a Durapore® membrane on a Waters Solvent Clarification Kit (P/N 85113) or other suitable membrane to remove microparticulate matter above 0.45 µm. A particulate-free mobile phase reduces the problem of plugged filters and column beds and preserves column life.
- Use vacuum filtration or sonication to remove dissolved gasses which could affect your mobile phase delivery system.
- Mobile phase solvents should be prepared by weight for maximum reproducibility.
- Use a Waters In-line Precolumn Filter (P/N84560) to obtain maximum column life.
- Filter particulate matter from samples to prevent excessive pressure buildup. Use Millex®-HV Filters (P/N 83996) or the Waters Sample Clarification Kit (P/N 26865) to filter samples.
- Samples should be disolved in acetonitrile/water (50:50) for optimal chromography.

III. OPERATION

CAUTION: Improper equilibration of this column may result in decreased column performance. Please read this section before attempting to use this column.

a. Column Equilibration

To equilibrate the column, set up a linear gradient from 100% water to 65 - 75% acetonitrile in water (depending on desired retention) at a rate of 0.5 ml/min for one hour. Then flow premixed (acetonitrile/water) mobile phase at final conditions for one additional hour at 1 ml/min.

b. Operation Precautions

Refer to the following list for operation precautions:

- Stay within a pH range of 2 -12.
- Filter all solvents. Avoid using turbid or cloudy mobile phases. Be sure that any solutions containing buffers, salts, and so on are compatible with the wetted surfaces of the column and equipment.
- Protect the column from vibration, mechanical shock and rapid changes in pressure. Column packings are based on a porous rigid polymer alignment. Any thermal, physical or chemical shock (such as changing mobile phases rapidly or high flow rates) can cause the particles to shift and may result in a loss of efficiency.
- Treat water with a Milli-Q® or equivalent system. De-ionized water is not acceptable because it contains organic compounds which alter column selectivity.
- Protect the column from rapid changes in mobile phase composition. DO NOT change the flow rate faster than 0.3 ml/min increments.
- Refer to Table 1 for recommended initial mobile phase compositions for various neutral oligosaccharides.

Table 1: Recommended Mobile Phase Compositions for Neutral Oliogiosaccharides

Oligosaccharide*	Retention Relative to N-Acetylglucosamine			
	% Acetonitrile			
	65	68	71	74
1	1.0	1.0	1.0	1.0
2	1.56	1.81	2.62	3.4
3	1.56	2.03	3.20	4.69
4	--	2.40	3.87	6.13
5	2.05	3.00	5.48	9.87
6	2.83	4.73	8.97	11.48

The table above represents retention relative to N-acetylglucosamine calculated as follows:

$$\text{Relative Retention} = \frac{k'x}{k' \text{ N-acetyglucosamine}}$$

The numbered structures refer to those found in Figure 4. Please note that the relative retention of these structures represent only approximate values and may be affected by variations in instrument configuration and mobile phase composition. They are provided as an aid to mobile phase selection and not as a diagnostic indicator of column performance.

c. Column Efficiency

Liquid chromatography columns have a finite life which is directly related to the care and use they receive. Column life is influenced by the number of injections, sample and solvent cleanliness, frequency of solvent changeover, and handling and storage procedures.

If you observe a change in the (1) retention of a particular compound, (2) resolution between two compounds, or (3) peak shape, take immediate steps to determine the reason for the changes. Until the cause of the change is determined, the results of any separation using the column must not be relied upon.

Follow generally accepted procedures for quality control and methods development when using these columns.

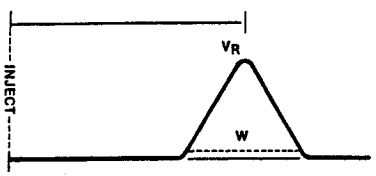
Waters columns are thoroughly tested in our quality control laboratories for adherence to our specifications. Variations in your results may occur depending on the equipment used, test sample makeup, mobile phase and equipment settings and conditions.

Note: Be sure to record results and instrument settings (and configurations) to allow exact reproduction and comparison in the future.

d. Column Testing

Waters uses the 5-sigma method shown in Figure 6 to measure column efficiency. Unlike the tangent method used to determine system efficiency, this stringent method considers naturally occurring peak asymmetry.

Figure 6: 5-Sigma Test Method



N = Column efficiency (plates)
V_R = Volume to peak apex (ml)
W = Volume at 4.4% of peak height (ml)

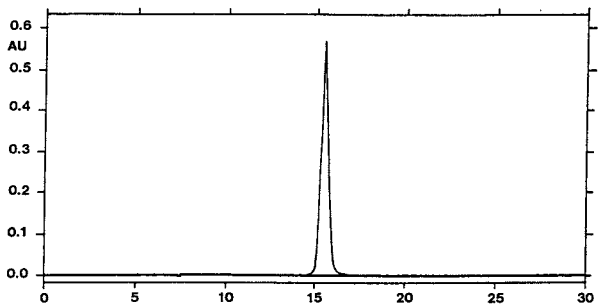
$$N = 25\left(\frac{V_R}{W}\right)^2$$

To aid in diagnosing potential column problems:

- Convert the column to acetonitrile/water (70:30) with a linear gradient over 1 hour at 0.5 m1/min.
- Equilibrate the column for 1 hour in premixed (w/w) acetonitrile/water (70:30) at a flow rate of 1 ml/min.
- Inject 10 µl of a 10 mg/ml solution of N-acetylglucosamine.
- Set detector to 214 nm.

Retention time will vary with absolute concentration of the solvents. The peak shape, however, should be similar to that seen in the chromatogram below.

Figure 7: Test N-Acetylglucosamine Chromatogram



Performing this test prior to. discussing the problem with your Waters representative will help in diagnosing the problem.

IV. MAINTENANCE AND TROUBLESHOOTING

The following sections explain how to store and troubleshoot the column.

a. Storage

Leaving the column unused for less than 72 hours does not generally require storage procedures.

Store the column in mobile phase or an acetonitrile/water 70:30 mixture. Return the column to its box with the end plugs firmly in place for storage. Allowing columns to dry out can result in poor chromatographic performance.

CAUTION: Glyco-Pak N columns are shipped in water. Ensure that the storage temperature does not go below 4 °C. The column may be damaged at temperatures below this value.

b. Troubleshooting

Use Table 2 to troubleshoot problems with your column.

Table 2: Column Problems and Solutions

Problem	Cause	Solution
Excess pressure buildup	• Filters plugged with Particulates • Sample pre-cipitates on column (sample not soluble in mobile phase)	• Clean in an ultrasonic bath or replace. • Always alter mobile phases and samples. • Slowly purge with a strong mobile phase that is both appropriate to dissolve the contaminate and compatible with the column.
Loss of resolution, broad peaks, low plate counts	• Contaminated column • Insufficient equilibration • Filters partially plugged	• Slowly purge with very strong mobile phase. • Continue equilibration • Replace or clean inlet and outlet filters in an ultrasonic bath.

V. APPENDIX

a. Warranty/Service Information

Waters Corporation staff of experienced service specialists provide maintenance assistance on both preventative and/or corrective levels. For complete information and assistance, please call Waters Service Department at 1-800-252-HPLC. For solutions to particular applications questions Waters team of technical support personnel are available to help you with specialized support. They may be contacted at 1-800-252-HPLC in Milford, MA.

Waters Corporation warrants its high performance liquid chromatography columns in accordance with the following terms and conditions:

Waters replace without cost any steel column that fails to perform satisfactorily if notified within 90 days from your receipt. Any column returned must have a Return Authorization Number granted by the waters Customer Service Department. Approval is subject to the following exclusions:

- Physical damage to the column because of misuse or abuse.
- Chemical damage to the packing material because of use with incompatible mobile phases or buffers, or at an incorrect pH.
- Physical damage to the packing material because of operation at incorrect temperatures or pressures.
- Particulate buildup or precipitation in the column or end fittings causing high internal pressure which has occurred because of improper mobile phase or sample filtration practices.

b. Ordering Information

Item	Part Number
Glyco-Pak N Column	WAT026725
Glyco-Pak DEAE Column	WAT026730
Sample Clarification Kit	WAT026865
Solvent Clarification Kit	WAT085113
In-line Precolumn Filter	WAT084560

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