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Poster Presentation

Preparative Scale Separations of Oligosaccharides on Polymeric HPLC Packing Materials

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PREPARATIVE SCALE SEPARATIONS OF OLIGOSACCHARIDES ON POLYMERIC HPLC PACKING MATERIALS

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Objective: To demonstrate multi-mode chromatographic approaches using polymeric HPLC packing materials in the preparative purification of oligosaccharides.

In elucidating oligosaccharide structures, identification of an unknown structure based on chromatographic retention time alone, is not convincing because of the immense number of possible structural isomers which may ce-elute with the compound of interest. Unequivocal structure determination of oligosaccharides requires the use of high field NMR and/or FAB-mass spectrometry. One drawback of these techniques is that they require a large quantity of a homogeneous sample. High-performance liquid chromatography (HPLC) has been used to purify large quantities of oligosaccharides in high purity from complex mixtures. Described in this paper are multi-mode chromatographic approaches, using stable polymeric columns, which demonstrate the usefulness of HPLC in the isolation and purification of large quanitities of oligosaccharides. Example one demonstrates the use of the Prep Glyco-Pak™ N column for the isolation and purification of neutral complex and high mannose oligosaccharides. Example two illustrates a loading optimization study on prototype Glyco-Pak II DEAE material packed in a glass AP column for the purification of sialylated oligosaccharides from fetuin. Example three illustrates the purification of chitin oligomers using the Glyco-Pak N and Ultrahydrogel™ DP columns.

INTRODUCTION:

In oligosaccharide structure elucidation work, no identification of an unknown structure based on chromatographic co-migration is convincing because of the immense number of possible structural isomers which might have identical retention times. Unequivocal structural determination of oligosaccharides requires the usage of high field NMR and/or FAB-mass spectrometry. One drawback of these techniques is that they require a large quantity of a homogeneous sample. High-performance liquid chromatography (HPLC) has been used to purify large quantities of oligosaccharides in high purity from complex mixtures. Described in this paper are several examples demonstrating the usefulness of HPLC with stable polymeric columns for the isolation and purification of large quanities of homogeneous oligosaccharides. Example one demonstrates the use of the Prep Glyco-Pak™ N column for the isolation and purification of neutral complex and high mannose oligosaccharides. Example two illustrates a loading optimization study on a prototype Glyco-Pak II DEAE in a glass AP column for the purification of sialylated oligosaccharides from fetuin. Example three uses a multi-chromatographic mode approach (using two polymeric columns) for the purification of chitin oligomers.

Prep Glyco-Pak™ N

Purification of high mannose and complex oligosaccharides

SAMPLE: Hydrazine released ovomucoid neutral oligosaccharides

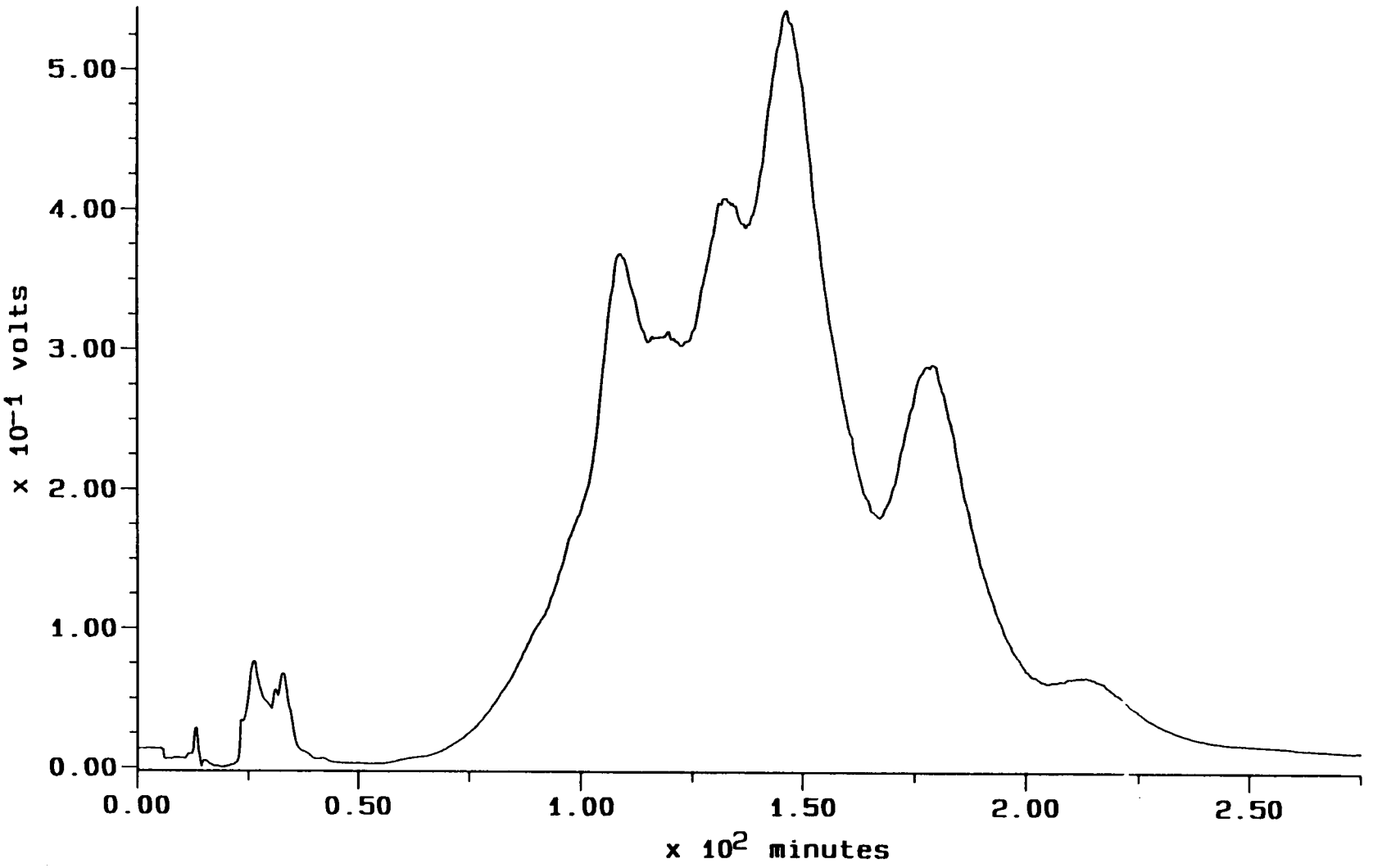
COLUMN: Prep Glyco-Pak™ N (22 x 600 mm)

ELUENT: Acetonitrile/Water (70:30)

FLOW RATE: 7.25 ml/min.

DETECTION: UV at 200 nm

INJECTION:: 20 mg of mixture



The maximum load on the analytical (7.8 x 300 mm) Glyco-Pak™ N column is 1 to 2 mg. Illustrated are scale-up isolations using the Prep Glyco-Pak N column (22 x 600 mm) at a flow rate of 7.25 ml/min. for neutral complex oligosaccharides (ovomucoid, Figures 1-3) and high mannose oligosaccharides (Figure 4). Based on scale up strategies, the load on the Prep Glyco-Pak N column is approximately 30 mg. Two injections of ovomucoid neutral complex oligosaccharides at sample loads of 20 mg and 40 mg show little deterioration of the analytical separation. Figure 4 shows the baseline separation of high mannose oligosaccharides on the Prep Glyco-Pak N column utilizing a sample load of 23 mg.

SAMPLE: Hydrazine released ovomucoid neutral oligosaccharides

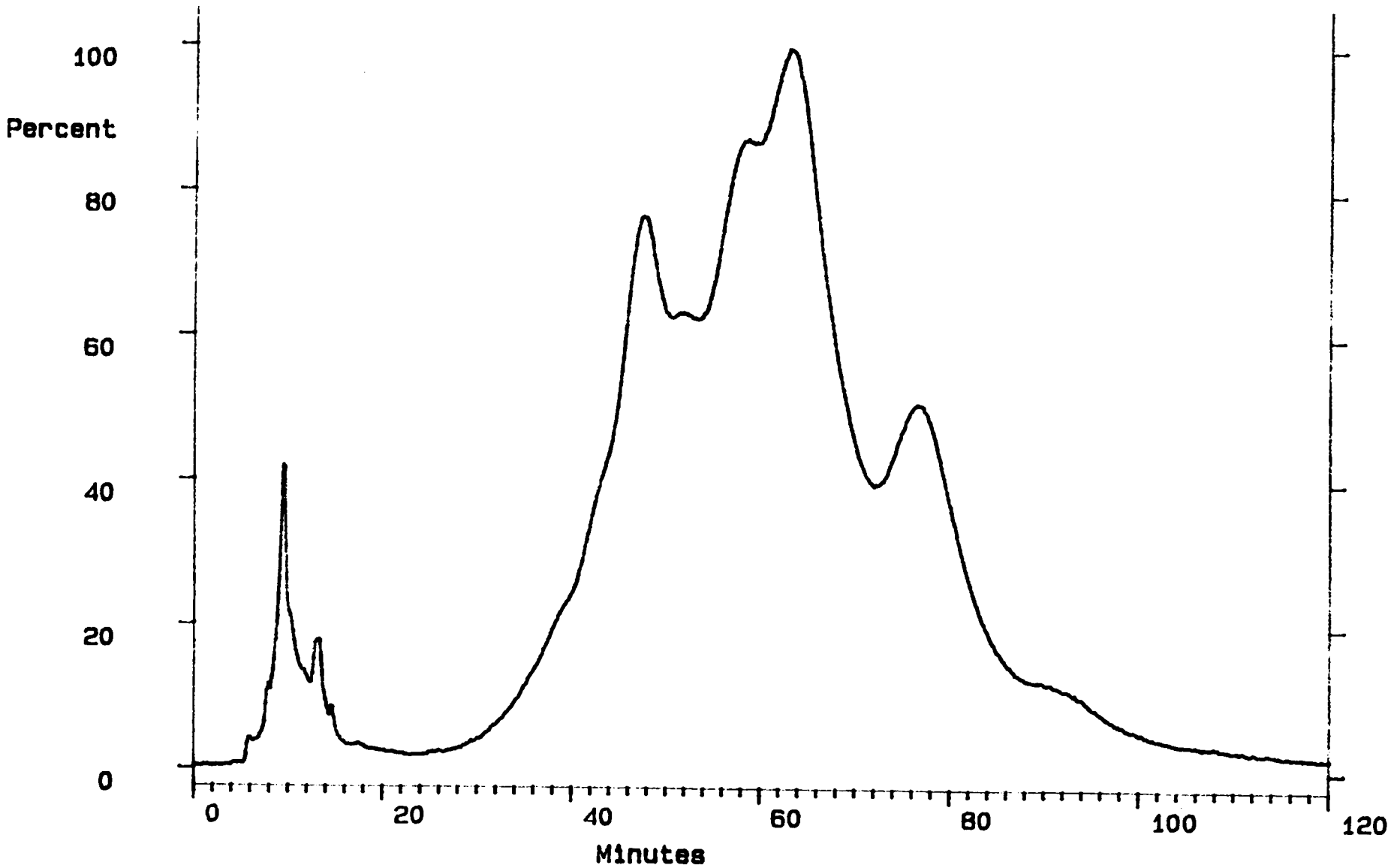
COLUMN: Glyco-Pak™ N (7.8 x 300 mm)

ELUENT: Acetonitrile/Water (70:30)

FLOW RATE: 1 ml/min.

DETECTION: UV at 197 nm

INJECTION:: 200 µg of mixture



SAMPLE: Reduced Endo H released high mannose oligosaccharides

COLUMN: Prep Glyco-Pak™ N (22 x 600 mm)

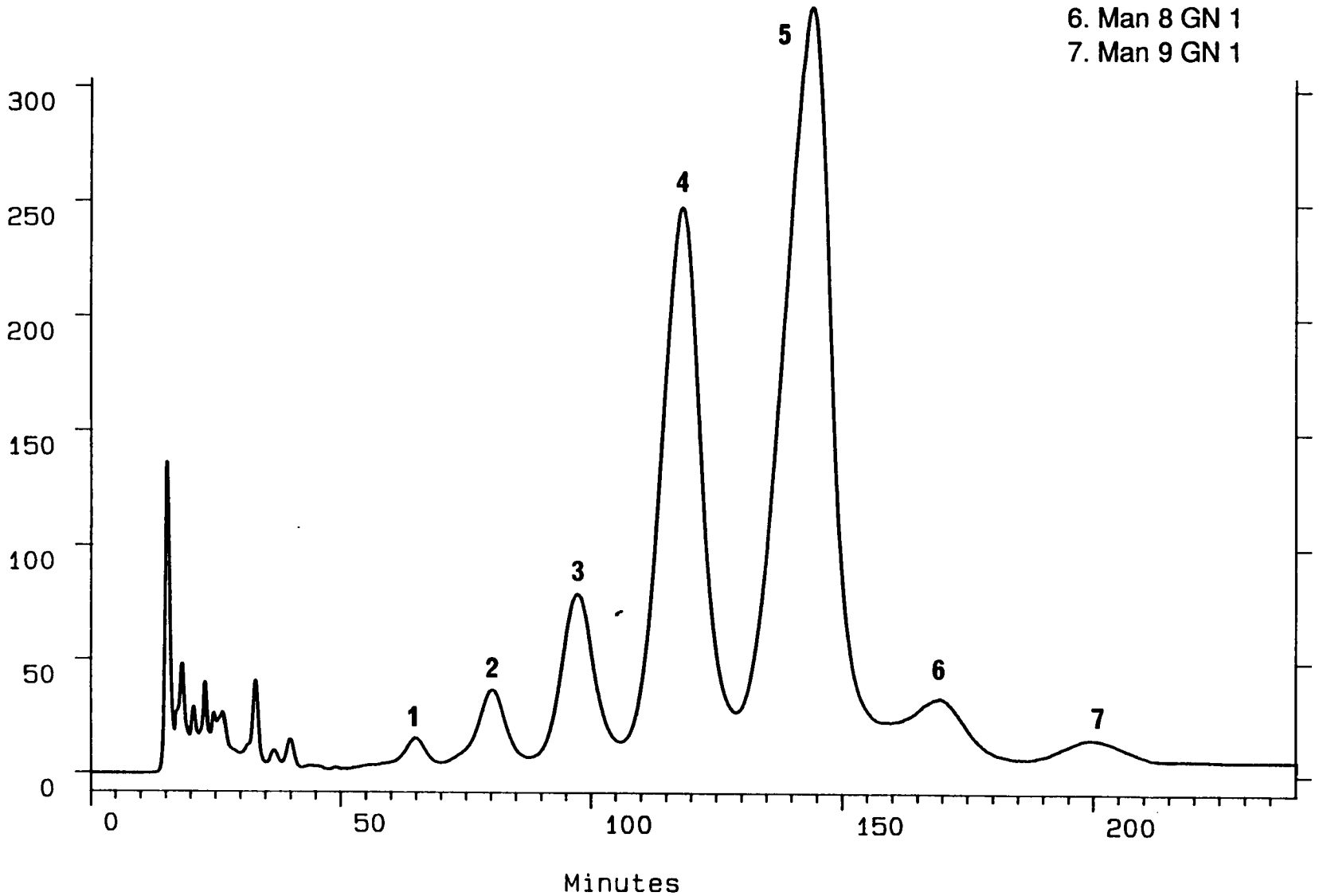
ELUENT: Acetonitrile/Water (70:30)

FLOW RATE: 7.25 ml/min.

DETECTION: UV at 200 nm

INJECTION: 23 mg of mixture

PEAK ID'S:
1. Man 3 GN 1
2. Man 4 GN 1
3. Man 5 GN 1
4. Man 6 GN 1
5. Man 7 GN 1
6. Man 8 GN 1
7. Man 9 GN 1



SAMPLE: Hydrazine released ovomucoid neutral oligosaccharides

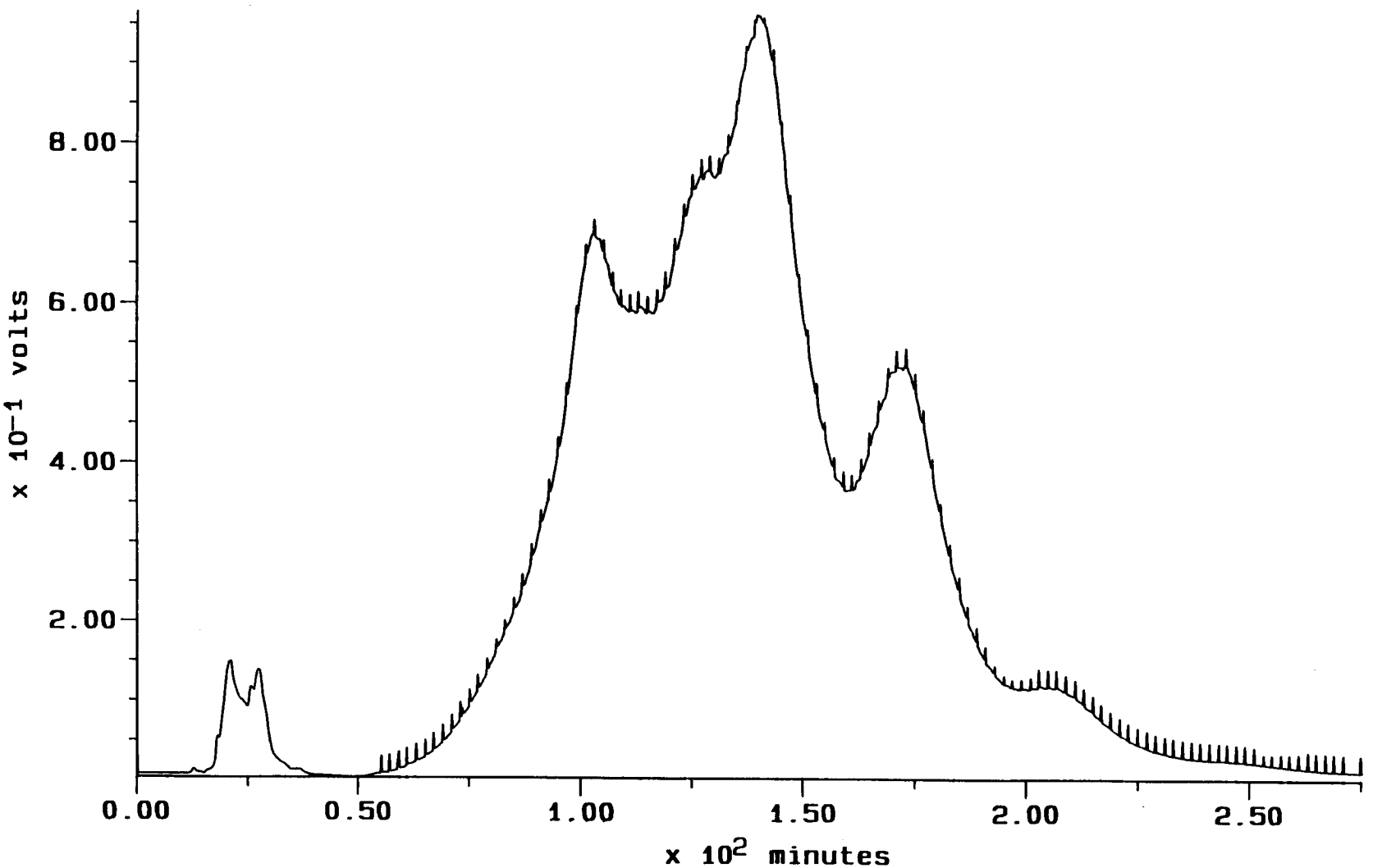
COLUMN: Prep Glyco-Pak™ N (22 x 600 mm)

ELUENT: Acetonitrile/Water (70:30)

FLOW RATE: 7.25 ml/min.

DETECTION: UV at 200 nm

INJECTION:: 40 mg of mixture



Loading optimization study on a prototype Glyco-Pak™ II DEAE in a glass AP column

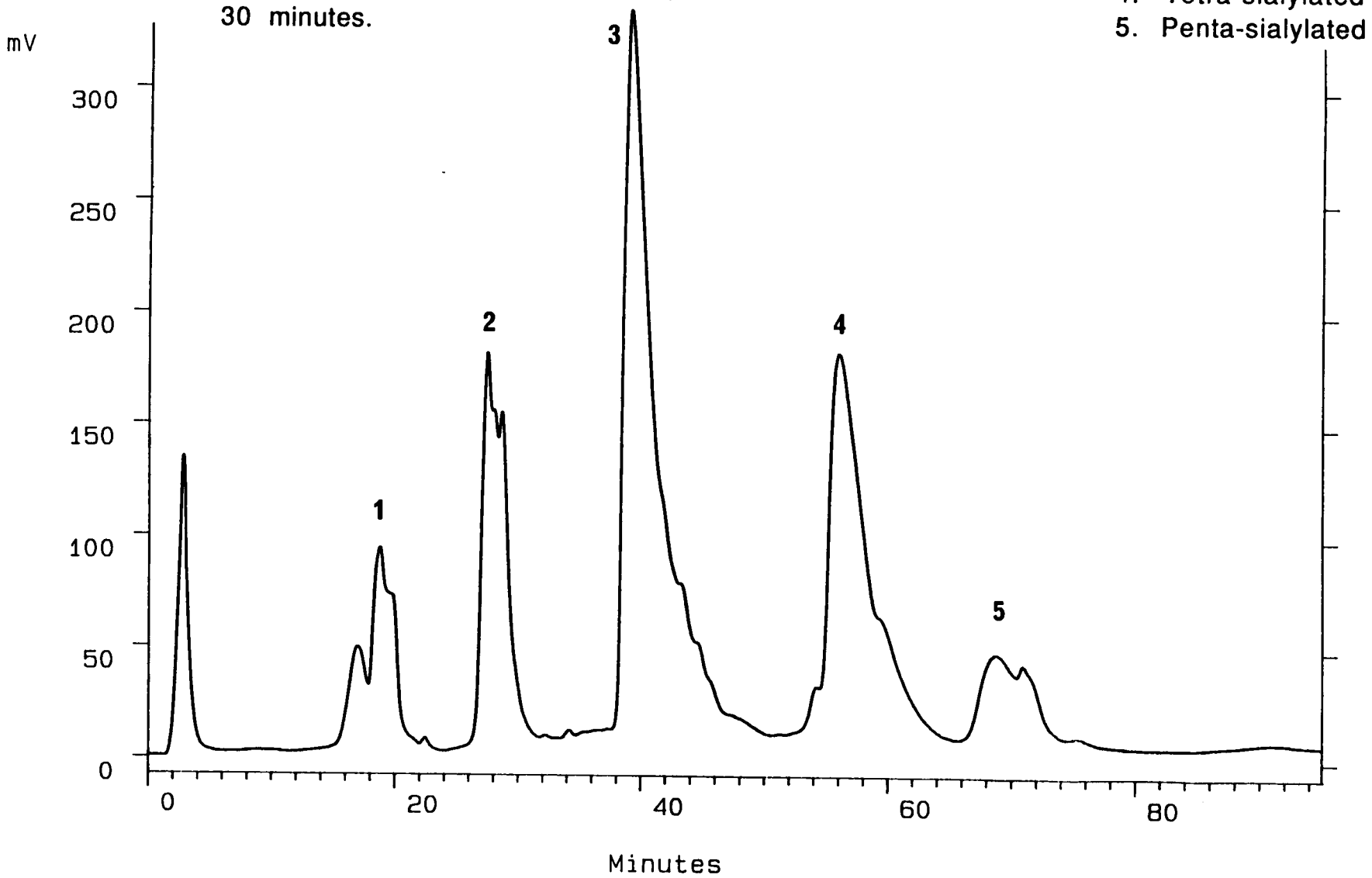
Purification of sialylated oligosaccharides from fetuin

In order to scale the sample load to larger columns for preparative isolation work, a loading study must be done on an analytical column. Illustrated (Figures 5-8) is a loading optimization study utilizing fetuin oligosaccharides on a prototype Glyco-Pak II DEAE in a glass AP column (10 x 100 mm). Figure 5 shows the analytical separation at 150 µg sample load. Figures 6 and 7 show sample loads of 5 and 10 mg respectively, with the 10 mg load showing signs of overload but still maintaining an acceptable separation. Figure 8 shows a sample overload of 50 mg with loss of resolution. From this study it is now known that a 10 mg load on the 10 x 100 mm column can be scaled to a 40 mg load on the 20 x 100 mm column and to 250 mg on the 50 x 100 mm column.

SAMPLE: Hydrazine released fetuin oligosaccharides
COLUMN: Prototype Glyco-Pak™ II DEAE in a glass AP column (10 x 100 mm)

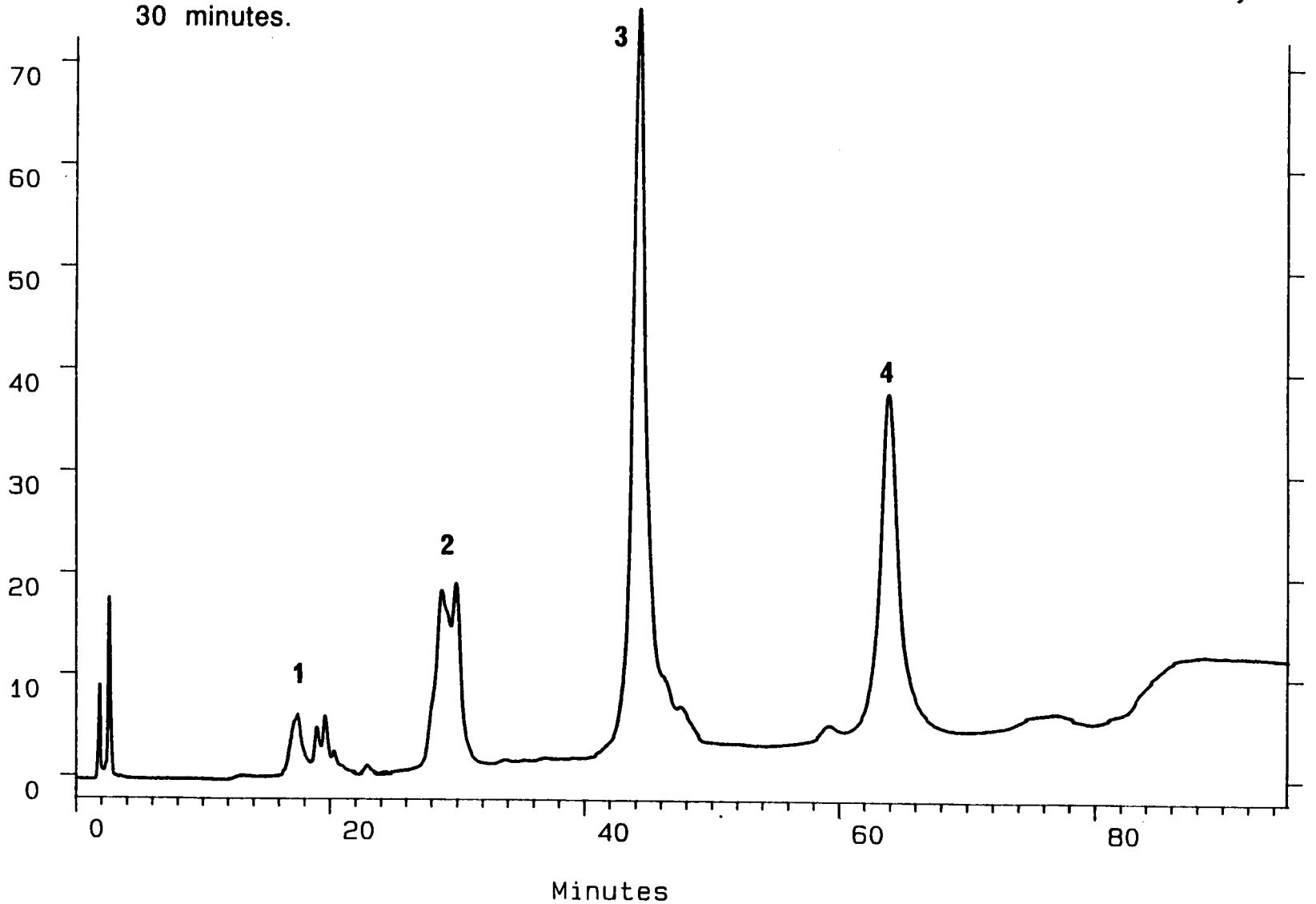
ELUENT A: Water
ELUENT B: 100 mM KH_2PO_4, pH 5.4
GRADIENT: Hold for 5 minutes at 0 mM B, then linear from 2 mM B to 50 mM B for 60 minutes, hold for 30 minutes.

FLOW RATE: 1.6 ml/min
DETECTION: UV at 220 nm
INJECTION: 5 mg of mixture
PEAK ID'S:
1. Mono-sialylated
2. Di-sialylated
3. Tri-sialylated
4. Tetra-sialylated
5. Penta-sialylated



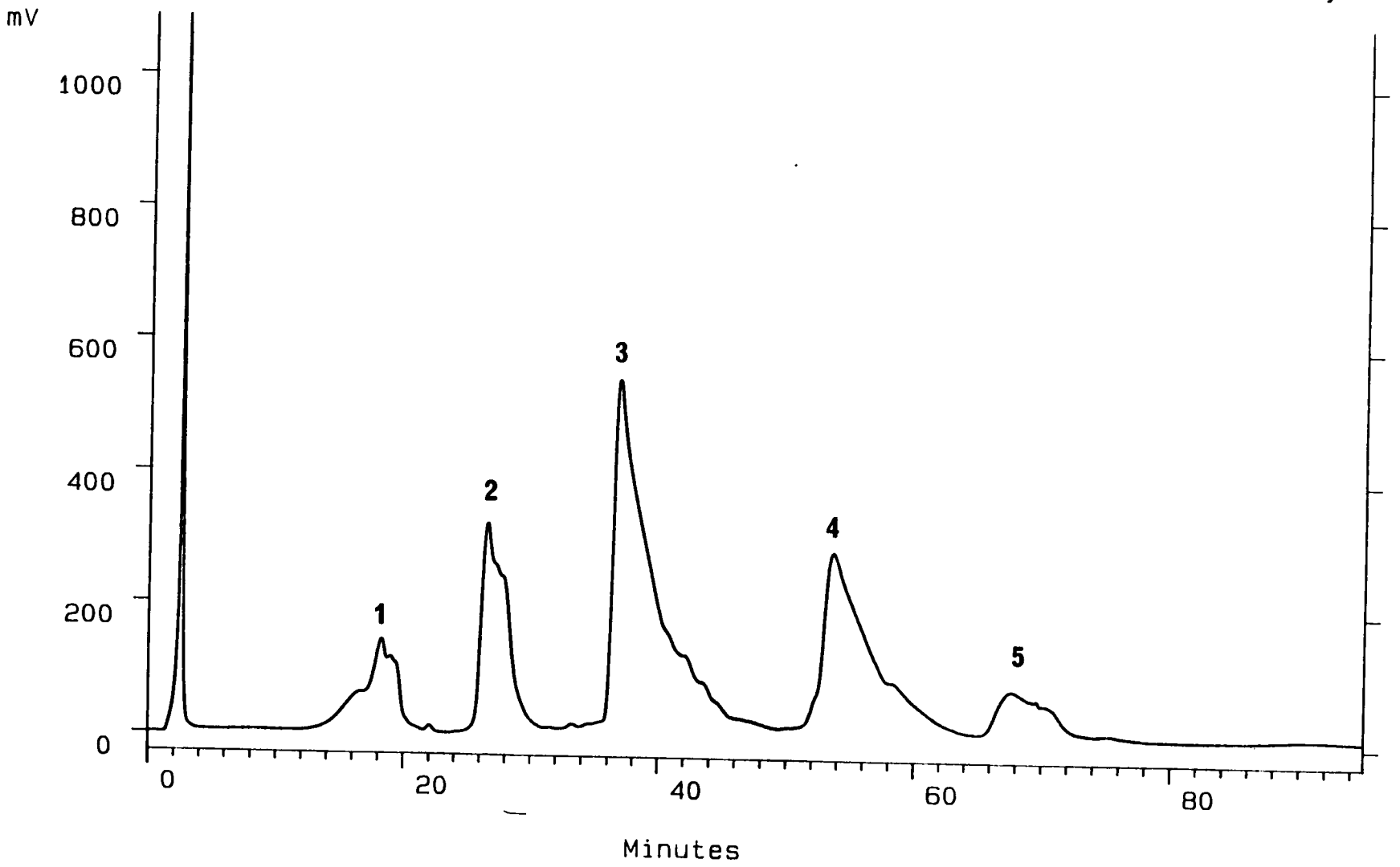
SAMPLE: Hydrazine released fetuin oligosaccharides
COLUMN: Prototype Glyco-Pak™ II DEAE in a glass AP column (10 x 100 mm)
ELUENT A: Water
ELUENT B: 100 mM KH_2PO_4, pH 5.4
GRADIENT: Hold for 5 minutes at 0 mM B, then linear from 2 mM B to 50 mM B for 60 minutes, hold for 30 minutes.

FLOW RATE: 1.6 ml/min
DETECTION: UV at 210 nm
INJECTION: 150 µg of mixture
PEAK ID'S:
1. Mono-sialylated
2. Di-sialylated
3. Tri-sialylated
4. Tetra-sialylated



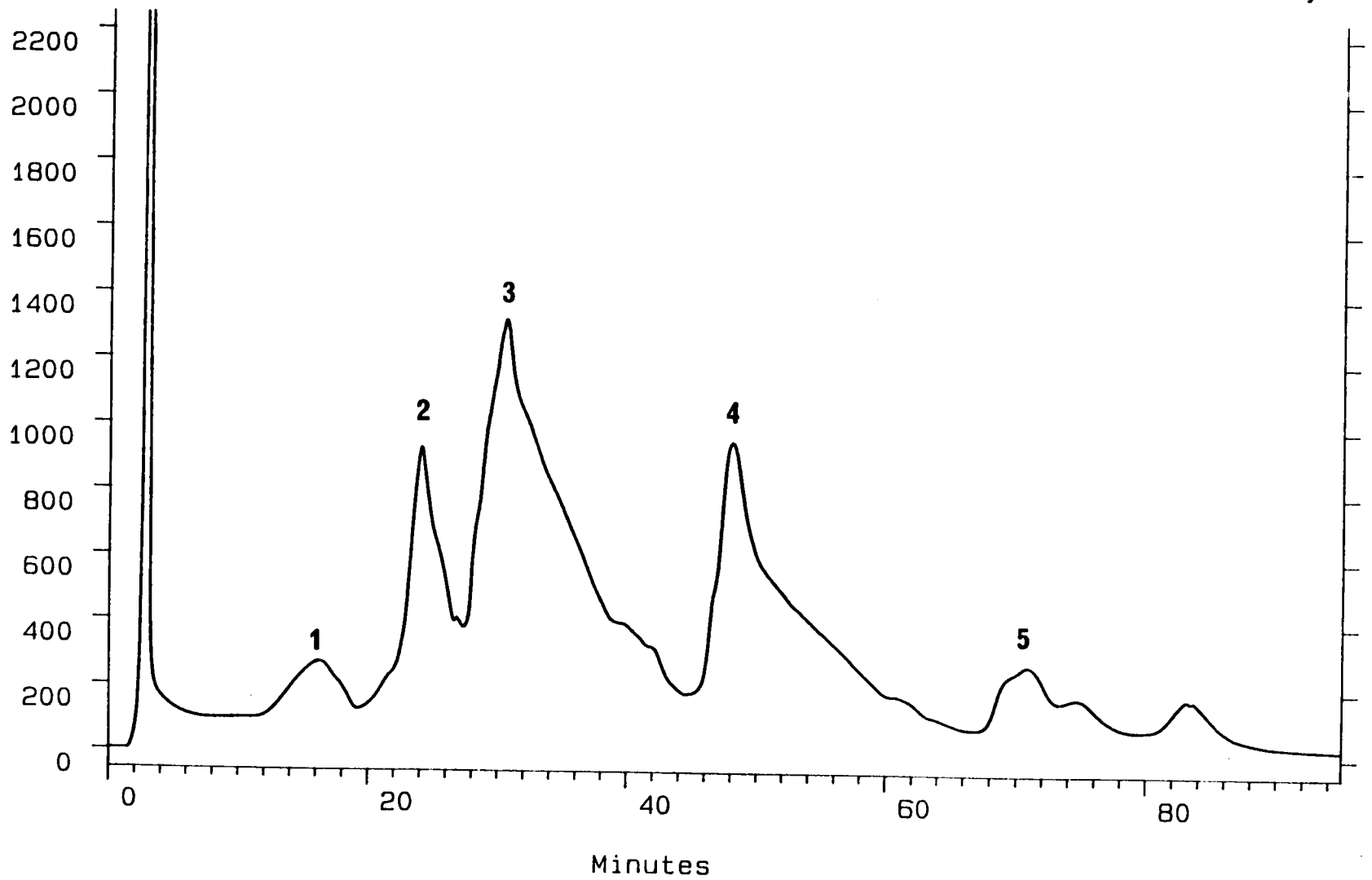
SAMPLE: Hydrazine released fetuin oligosaccharides
COLUMN: Prototype Glyco-Pak™ II DEAE in a glass AP column (10 x 100 mm)
ELUENT A: Water
ELUENT B: 100 mM KH_2PO_4, pH 5.4
GRADIENT: Hold for 5 minutes at 0 mM B, then linear from 2 mM B to 50 mM B for 60 minutes, hold for 30 minutes.

FLOW RATE: 1.6 ml/min
DETECTION: UV at 220 nm
INJECTION: 10 mg of mixture
PEAK ID'S:
1. Mono-sialylated
2. Di-sialylated
3. Tri-sialylated
4. Tetra-sialylated
5. Penta-sialylated



SAMPLE: Hydrazine released fetuin oligosaccharides
COLUMN: Prototype Glyco-Pak™ II DEAE in a glass AP column (10 x 100 mm)
ELUENT A: Water
ELUENT B: 100 mM KH_2PO_4, pH 5.4
GRADIENT: Hold for 5 minutes at 0 mM B, then linear from 2 mM B to 50 mM B for 60 minutes, hold for 30 minutes.

FLOW RATE: 1.6 ml/min
DETECTION: UV at 220 nm
INJECTION: 50 mg of mixture
PEAK ID'S:
1. Mono-sialylated
2. Di-sialylated
3. Tri-sialylated
4. Tetra-sialylated
5. Penta-sialylated

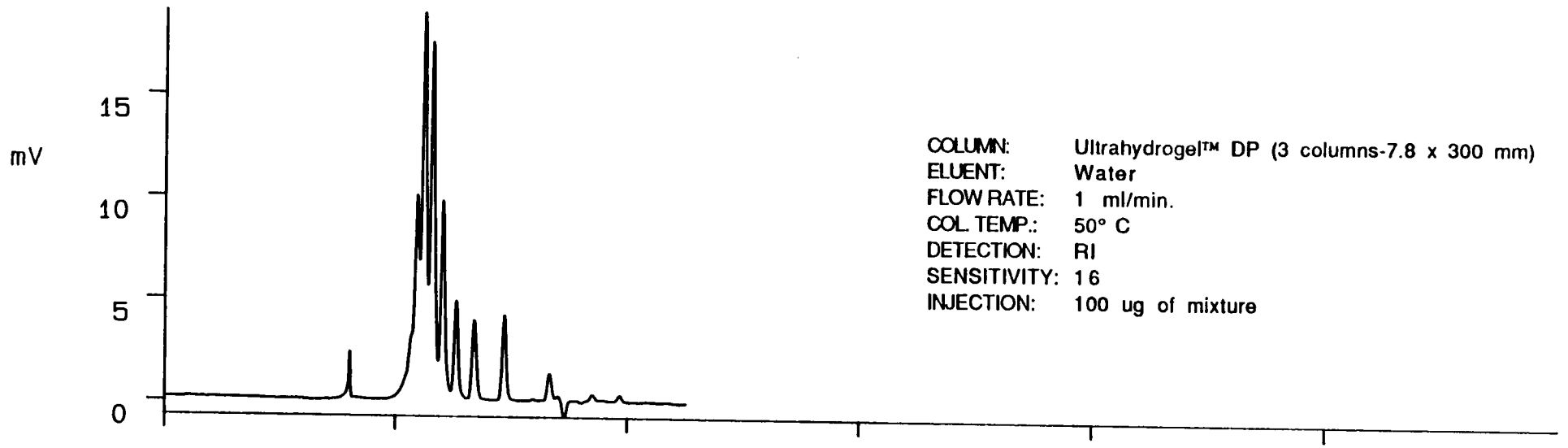


Illustrated is the isolation and purification of chitin oligosaccharides (Figures 9-15) utilizing a multiple chromatographic separation mode approach. Ultrahydrogel™ DP (aqueous size exclusion chromatography) and Glyco-Pak™ N (acetonitrile/water partition chromatography) columns have been used to evaluate chitin samples prior to and after purification on a Prep Glyco-Pak N column. Figures 9 and 10 respectively show the separation of crude chitin and a fraction (Fraction A) of the higher oligomers using a bank of three Ultrahydrogel DP columns and on an analytical Glyco-Pak N column. With the Ultrahydrogel columns, the larger oligomer elutes first (Peak 6 is a larger oligomer than Peak 5). With the Glyco-Pak N column the reverse is true, the larger oligomer (Peak 6) elutes later than the smaller oligomer (Peak 5). Because the resolution of the oligomers is best on the Glyco-Pak N column, 8 mg of Fraction A chitin oligomers were purified on a Prep Glyco-Pak N column (Figure 11). Inorder to determine the purity of the materials isolated, Figures 12 - 15 show the analysis of the fractions pooled to represent peaks 5 and 6 on the Ultrahydrogel DP columns and on the Glyco-Pak N column. Figure 12 shows that on the Ultrahydrogel DP columns, Peak 6 appears to be pure. However, the analysis of Peak

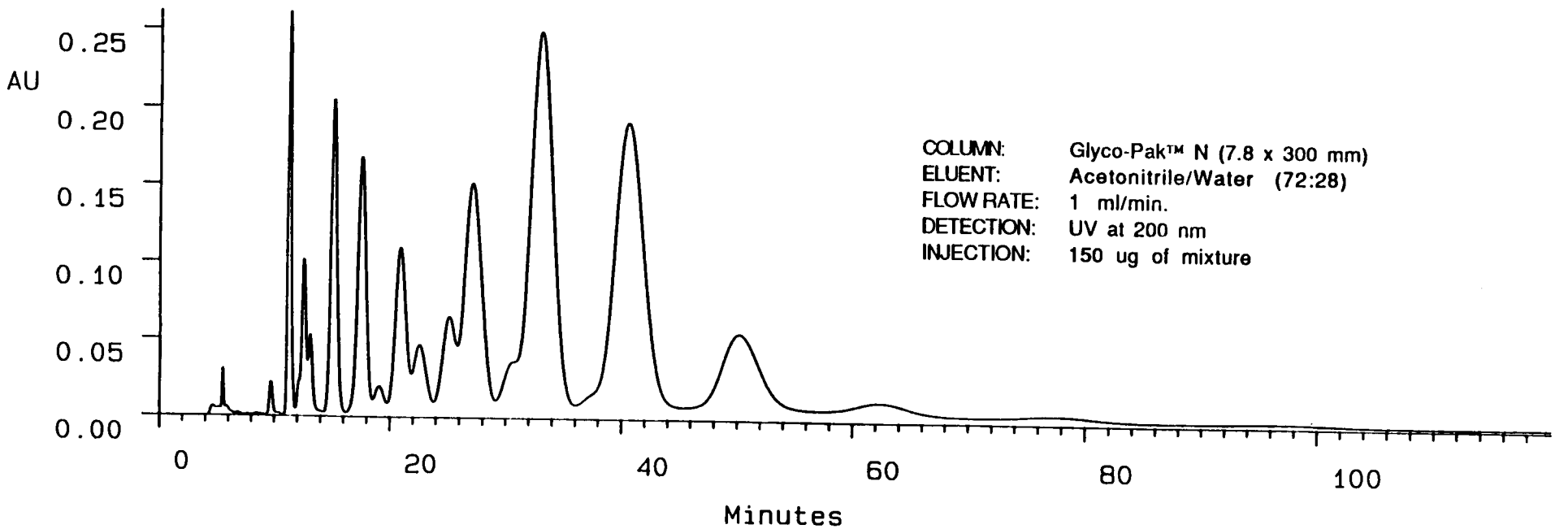
6 on the Glyco-Pak N column (Figure 13) shows that there is a small amount of Peak 5 still present. Figure 14 shows the analysis of Peak 5 with the Ultrahydrogel DP columns which is still contaminated with a small quantity of a larger oligomer (possibly Peak 6). Figure 15 shows that Peak 5 is also contaminated with a small quantity of a smaller oligomer (present as a shoulder). This multiple column approach, which utilizes two different selectivities (size exclusion and partition), illustrates the strength of using more than one chromatographic mode for the fractionation and purification of oligosaccharides prior to structure elucidation.

Preparative isolation of chitin oligosaccharides

Chitin Oligosaccharides

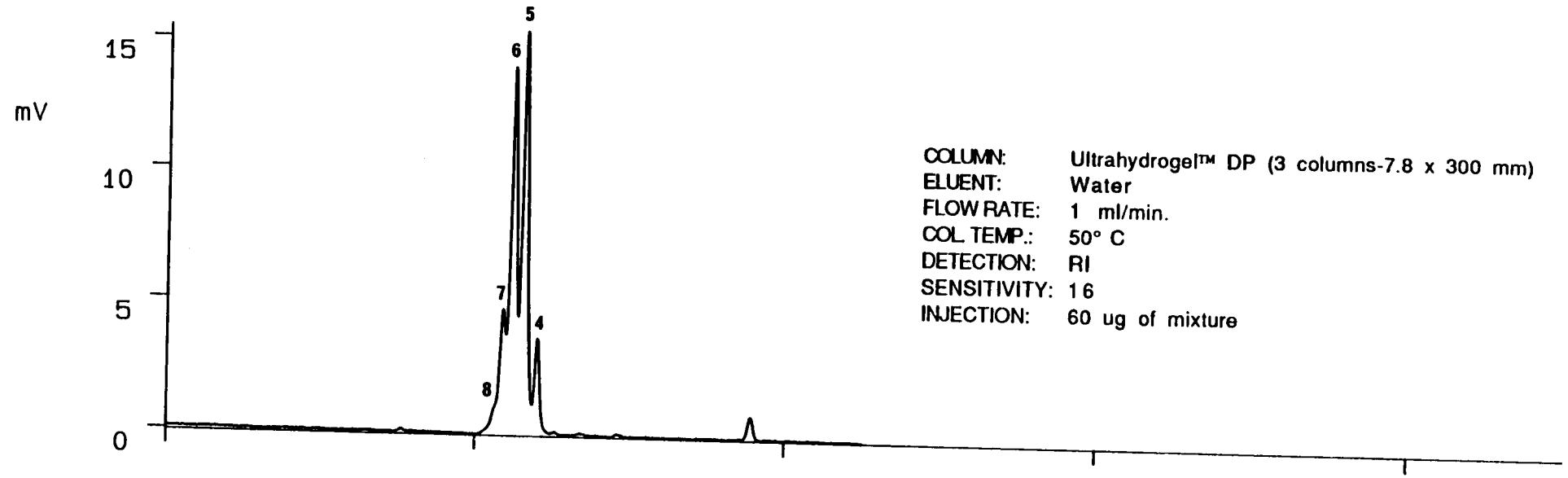


COLUMN: Ultrahydrogel™ DP (3 columns-7.8 x 300 mm)
ELUENT: Water
FLOW RATE: 1 ml/min.
COL. TEMP.: 50° C
DETECTION: RI
SENSITIVITY: 16
INJECTION: 100 ug of mixture

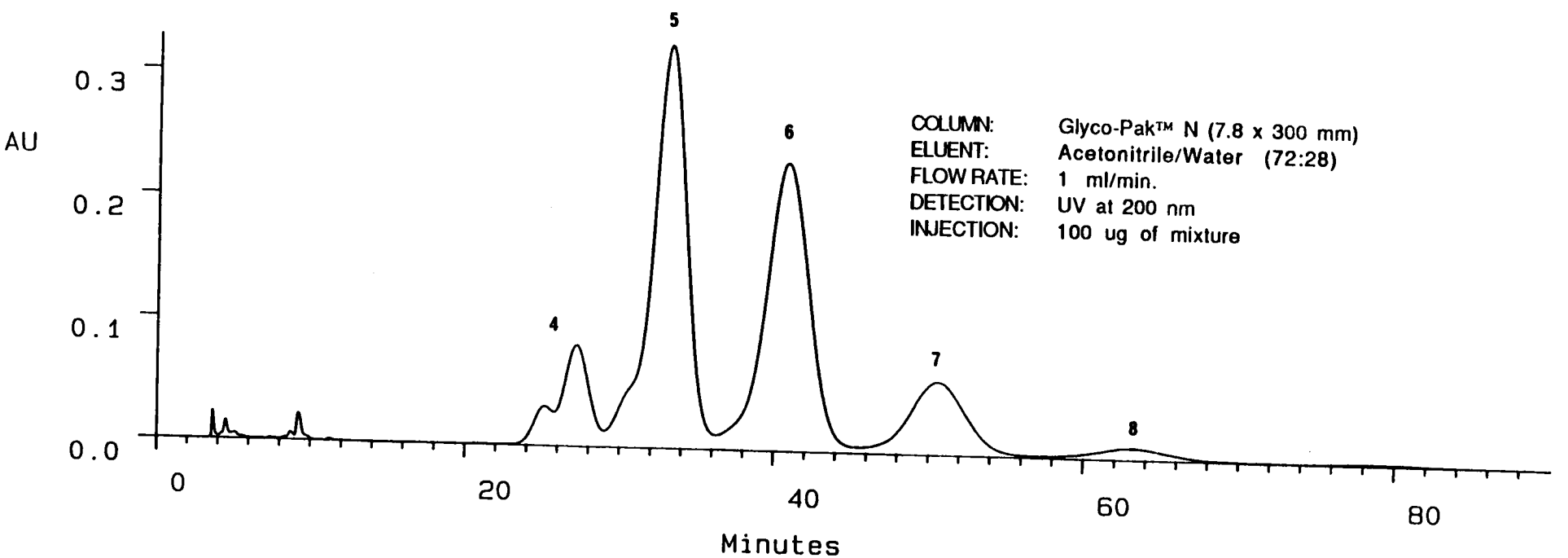


COLUMN: Glyco-Pak™ N (7.8 x 300 mm)
ELUENT: Acetonitrile/Water (72:28)
FLOW RATE: 1 ml/min.
DETECTION: UV at 200 nm
INJECTION: 150 ug of mixture

Chitin Oligosaccharides
Fraction A



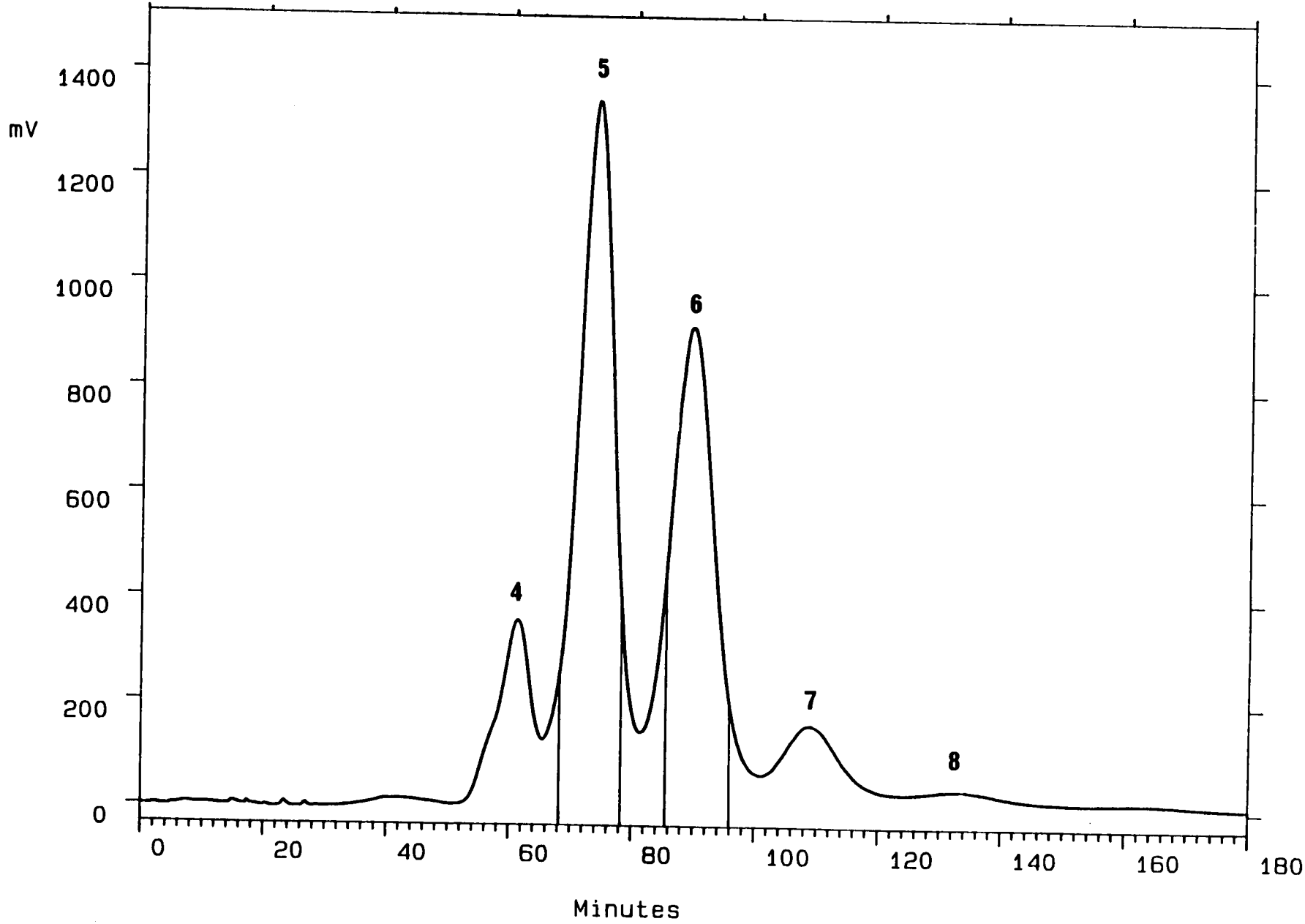
COLUMN: Ultrahydrogel™ DP (3 columns-7.8 x 300 mm)
ELUENT: Water
FLOW RATE: 1 ml/min.
COL. TEMP.: 50° C
DETECTION: RI
SENSITIVITY: 16
INJECTION: 60 ug of mixture



COLUMN: Glyco-Pak™ N (7.8 x 300 mm)
ELUENT: Acetonitrile/Water (72:28)
FLOW RATE: 1 ml/min.
DETECTION: UV at 200 nm
INJECTION: 100 ug of mixture

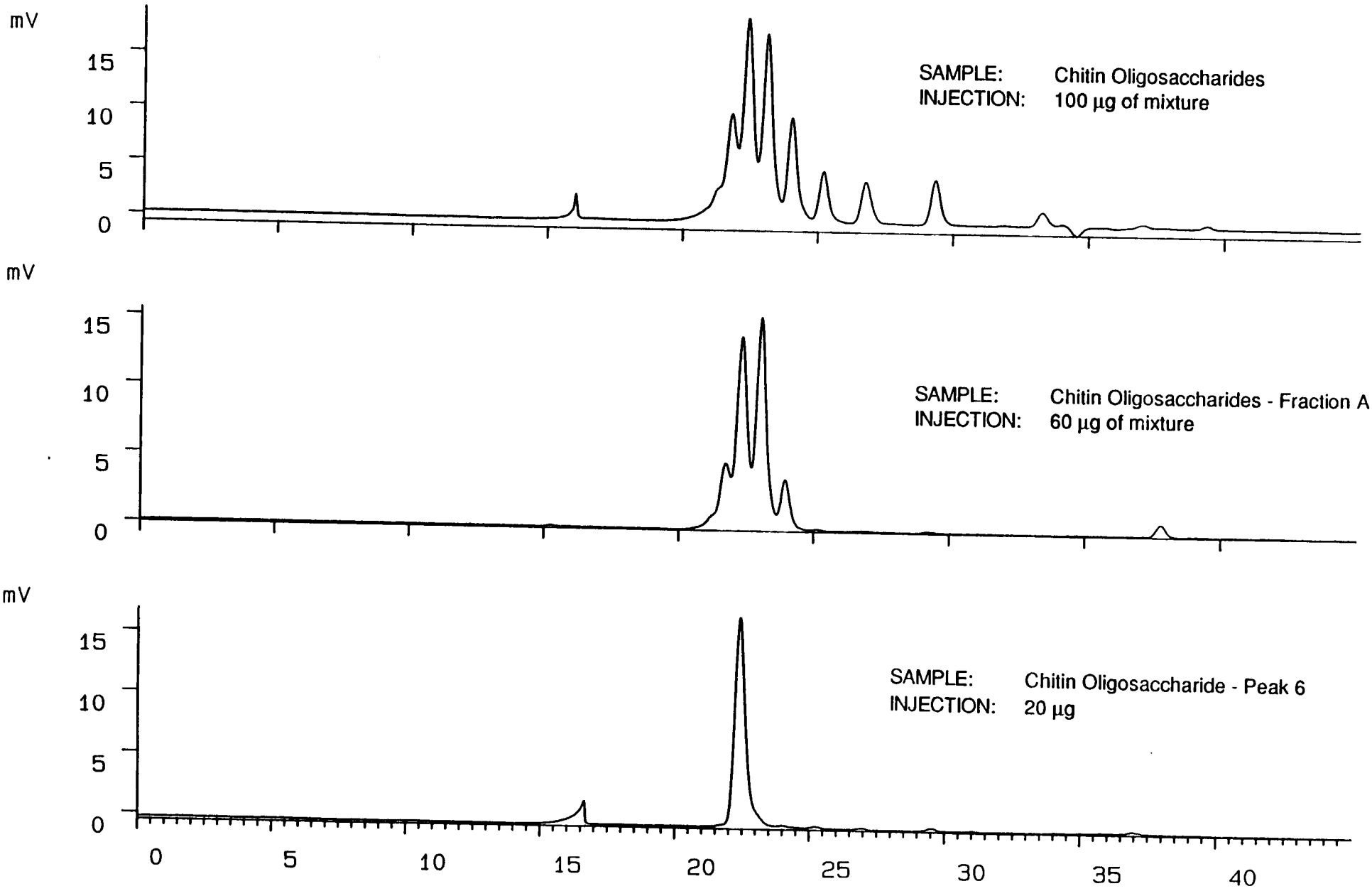
SAMPLE: Chitin Oligosaccharides Fraction A
COLUMN: Prep Glyco-Pak™ N (22 x 600 mm)
ELUENT: Acetonitrile/Water (72:28)
FLOW RATE: 7.25 ml/min.

DETECTION: UV at 200 nm
INJECTION:: 8 mg of mixture



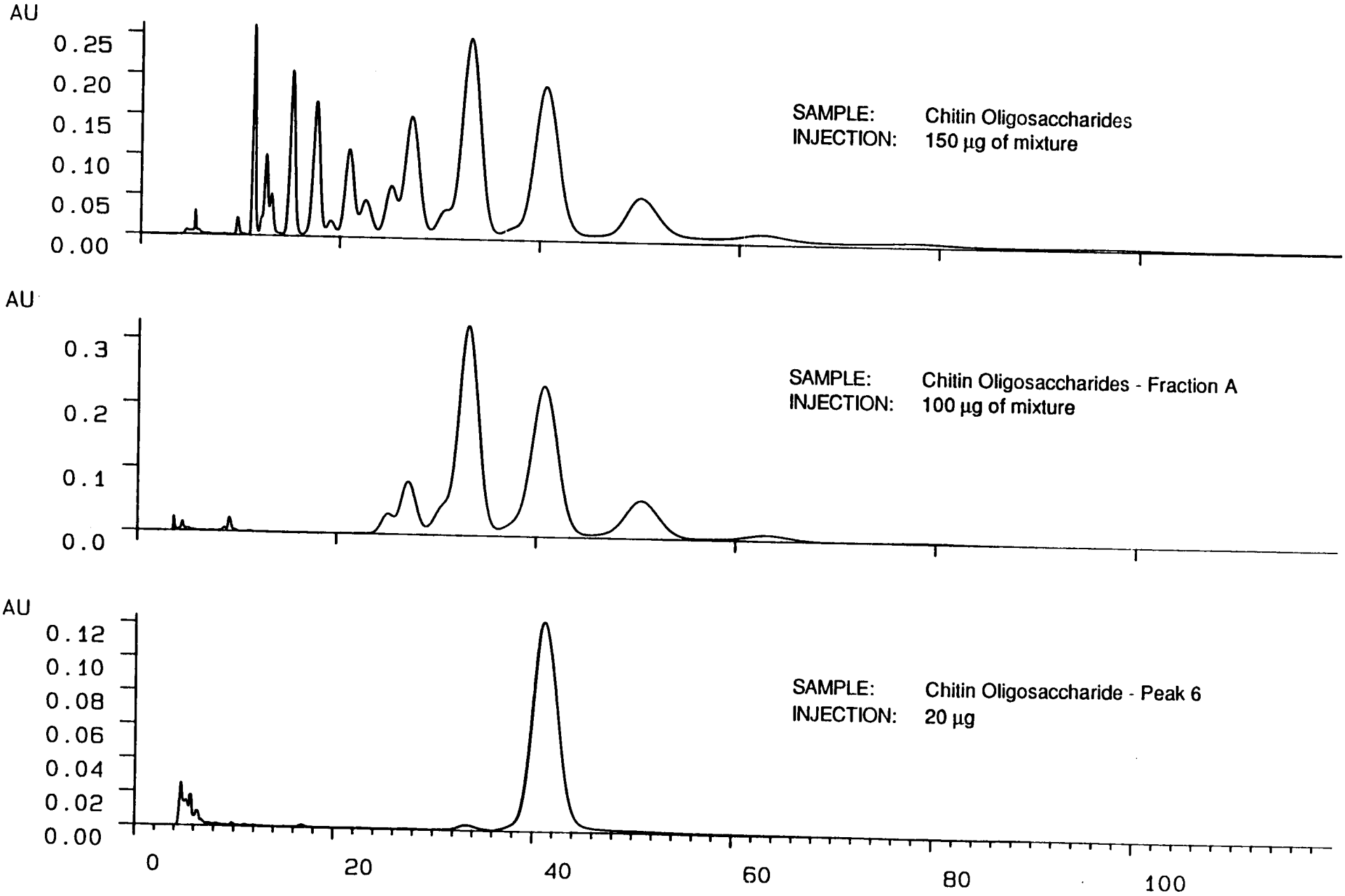
COLUMN: Ultrahydrogel™ DP (3 columns-7.8 x 300 mm)
ELUENT: Water
FLOW RATE: 1 ml/min.
COL. TEMP.: 50° C

DETECTION: RI
SENSITIVITY: 16



COLUMN: Glyco-Pak™ N (7.8 x 300 mm)
ELUENT: Acetonitrile/Water (72:28)
FLOW RATE: 1 ml/min.

DETECTION: UV at 200 nm



COLUMN: Ultrahydrogel™ DP (3 columns-7.8 x [redacted] mm)
ELUENT: Water
FLOW RATE: 1 ml/min.
COL. TEMP.: 50° C

DETECTION: RI
SENSITIVITY: 16