Size-Exclusion Chromatography of Polysaccharides with Pulsed Amperometric Detection (PAD)

INTRODUCTION

Size-exclusion chromatography is used in carbohydrate research to determine molecular weight distributions of polysaccharides. A variety of detection methods have been used, among them viscometry,[1] low-angle laser light scattering (LALLS)[1], refractive index,[2] ultraviolet/visible absorbance,[3-4] fluorescence,[5] and precision differential refractometry coupled with ultraviolet detection.-[6] This application note focuses on pulsed amperometric detection, a method that provides sensitive and specific detection of carbohydrates.

Size-exclusion columns separate biological macromol ecules by hydrodynamic size. The Zorbax® size-exclusion columns used in this study contain highly stable spherical, silica-based packings with diol functional groups. The packings are stable over a pH range of 3.0 to 9.0. The Zorbax SE-250 separates globular proteins by molecular size over a range of 5000 to 300,000 daltons. The Zorbax SE-450 separates globular proteins by molecular size over a range of 15,000 to 1,000,000 daltons. The exclusion volume for these columns is about 6 mL, while the included volume is around 13 to 15 mL. (Some volumes on the chromatograms shown in this application note are different from those given by the column manufacturer. These differences may be due to the non-size-exclusion effects caused by the weak ionic strength of the eluent.[7])

Carbohydrates are not globular but rather linear or branched, so their behavior differs greatly from that of proteins under native conditions on size-exclusion columns. Fractionated pullulan standards were used to determine the separation range of linear carbohydrates. Pullulan is a straight chain of maltotriose units.

Carbohydrates can be classified by several categories based on their structure: linear or branched, type of linkage, and type of sugar residue. Table 1 on page 2 presents some common polysaccharides and their structural information.[8]

EQUIPMENT

Dionex chromatographic system consisting of:

Gradient Pump

Liquid Chromatography Module

Pulsed Electrochemical Detector

or Pulsed Amperometric Detector

Eluent Degas Module

Postcolumn reagent delivery system

AI-450 Chromatography Workstation or other data acquisition system

Table 1 Structural Summary of Selected Polysaccharides				
Carbohyrate	**Structure**	**Linkage**	**Sugar Unit**	**Molecular Weight**
Agar	Branched	alt α-(1-3) β-(1-4)	D- & L-galactose Some esterified sulfonic acid	Variable
Chitosan	Mostly linear	β-(1-4)	N-acetyl D-glucosamine	Variable
Dextran	Branched	Straight α-(1-6), branch at 1-2, 1-3, 1-4, or 1-6	D-glucose	15,000 – 20,000
Gum arabic (acacia)	Branched	Complex branching	D-galactose, rhamnose, arabinose, D-glucuronic acid	Variable
Gum ghatti	Branched	Complex branching	L-arabinose, D-galactose, D-mannose, D-xylose, D-glucuronic acid, (10:6:2:1:2) & trace amounts of 6-deoxyhexose	12,000
Inulin	Linear	β(2-1)	D-fructose, D-glucose	5,000
Locust bean gum	Linear chain of mannose branched every 4th mannose to 1 galactose		Mannose Galactose	310,000
Mannan			Mannose	Variable
Pectin	Branched	α,β-(1-4) straight (1-2) branched	Methyl ester of D-polygalacturonate sequences interrupted with L-rhamnose and side chains of D-galactose, L-arabinose, D-xylose and L-fucose	20,000 – 400,000
Pullulan	Linear	α-(1-4) α-(1-6)	Maltotriose Between triose	Variable
Starch	Linear (amylose) Branched (amylopectin)	α-(1-4) straight α-(1-6) branched	Glucans made of D-glucose units	Variable, amylose (350 μ) Amylopectin (25 μ)

REAGENTS AND STANDARDS

Glacial acetic acid

Sodium acetate, trihydrate

Sodium hydroxide solution, 50% w/w, low carbonate

Pullulan standards (can be obtained from JM Science, Shodex, or Megazyme):

Std.	MW Avg.*	Std.	MW Avg.*
P-5	5,800	P-100	100,000
P-10	12,200	P-200	186,000
P-20	23,700	P-400	380,000
P-50	48,000	P-800	853,000

* in Daltons

Agar	Inulin
Chitosan	Locust Bean Gum
Dextran	Mannan
Gum Arabic (Acacia)	Pectin
Gum Ghatti	Starch, soluble

CONDITIONS

Column: Zorbax BioPlus™ SE-250 or SE-450

Sample Loop Volume: 50 μL

Eluent: 10 mM Acetate buffer

Flow Rate: 1.0 mL/min

Postcolumn Reagent: 300 mM Sodium hydroxide

Expected Operating Pressure: 600–900 psi (4–6 MPa)

Detection: Pulsed amperometry, gold working electrode;
PED program no. 1, or
PAD settings as follows:

t (ms)	E (volts)
480	+0.05
120	+0.60
60	-0.60

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent: 10 mM Acetate Buffer

Dilute 0.28 mL of concentrated glacial acetic acid in 18-MΩ water. Add 0.68 g sodium acetate trihydrate and dissolve. Dilute to 1.0 L with 18-MΩ water.

Postcolumn Reagent: 300 mM Sodium Hydroxide

Dilute 15 mL of sodium hydroxide solution (50%) to 1 L with 18-MΩ water.

Pullulan Standards

Dissolve 50 mg of pullulan standard fraction in 18-MΩ water. Dilute to 100 mL with 18-MΩ water. Do not stir or heat the solution. Let the pullulan particles hydrate overnight at 0 to 4 ºC. Then carefully swirl the container to mix before injection.

Polysaccharides

Add 1 g to 100 mL of 18-MΩ water. Stir overnight at room temperature. Dilute stock to 1/10 or 1/100, as appropriate. Chitosan was dissolved in the acetate buffer eluent instead of 18-MΩ water. For agar, chitosan, and locust bean gum, heat was applied to facilitate dissolution.

RESULTS AND DISCUSSION

Pulsed amperometric detection (PAD) utilizes a repeating sequence of three potentials, which are applied for specific durations (see Conditions section). If only a single potential were used, the peak heights would steadily decrease as the electrode surface became fouled. PAD is most sensitive for carbohydrates at a pH of 12 or greater, but a mobile phase of this pH would quickly destroy the silica-based size-exclusion columns. Instead, a buffered mobile phase is used and sodium hydroxide added postcolumn to raise the pH. Using the pulsed conditions in this note, PAD is specific for carbohydrates. For further details concerning carbohydrate determination with PAD, please refer to Dionex Technical Note 20.[9]

Using pullulan standards, the molecular weight range for straight chain polysaccharides was established. The SE-250 separation range is 5,800 to 100,000 daltons (Figure 1) and the SE-450 separation range was 5,800 to 380,000 daltons (Figure 2). This range is significantly less than the range for globular molecules such as proteins.[10] These columns, as with any size-exclusion technology, can resolve to baseline compounds whose molecular weights differ two-fold. If the molecular weights differ by only a few hundred daltons, the compounds appear as a broad peak, which allows a qualitative determination of the distribution of sizes.

Figures 3 and 4 show molecular weight versus retention time calibration curves for the SE-250 and SE-450, respectively. Each figure shows the calibration curve for globular proteins as well as the calibration curve for the straight-chain pullulan standards. The elution behavior of the more highly branched polysaccharides resembles that of globular proteins on these columns.

Agar, gum arabic, gum ghatti, inulin, and starch were of a size small enough to exhibit a single peak near the included volume on the SE-450 column, but heterogeneous enough to exhibit several peaks (not baseline resolved) on the SE-250. Chromatograms of gum ghatti (Figure 5) are shown as examples. Locust bean gum appears as a series of five peaks on the SE-250. Four of the peaks (not baseline resolved) are of low molecular weight, and one small peak is excluded from the column. On the SE-450, the low-molecular weight peaks are unresolved, while the high-molecular weight peak is still excluded (Figure 6). Chitosan, mannan, and pectin exhibit peaks only on the SE-450. Chitosan is shown in Figure 7 as an example.

Dextran has a very narrow molecular weight distribution, and thus gives one peak on each column. Dextran on the SE-450 is shown in Figure 8.

CONCLUSION

The SE-250 is better able to resolve small molecular weight differences of the smaller polysaccharides (see Figures 5 and 6), while the SE-450 is better suited to resolving polysaccharides of higher molecular weight (compare Figures 1 and 2 for the larger pullulan fractions). In general, linear polysaccharides exhibit shorter retention times than branched polysaccharides of comparable molecular weights, probably because of their larger hydrodynamic volumes.

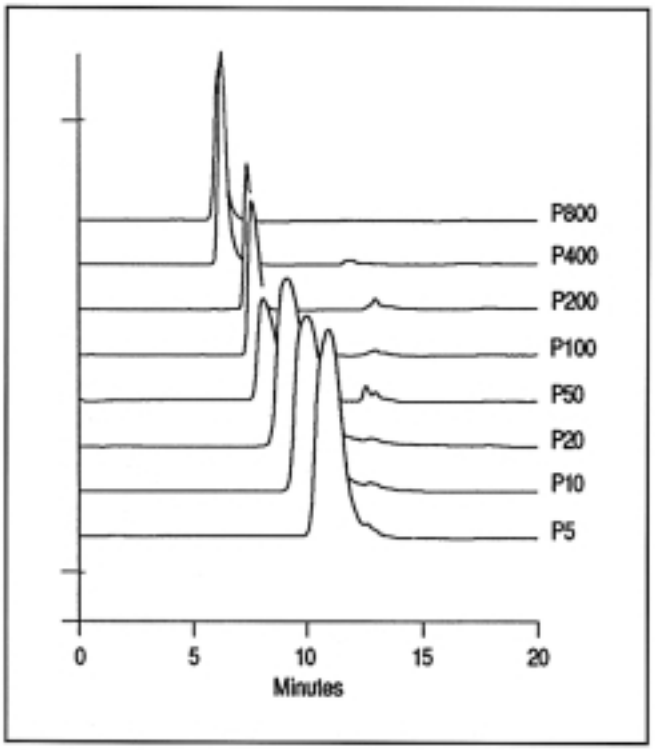

Figure 1. Size-exclusion chromatographic profile of pullulan fractions on the SE-250 column.

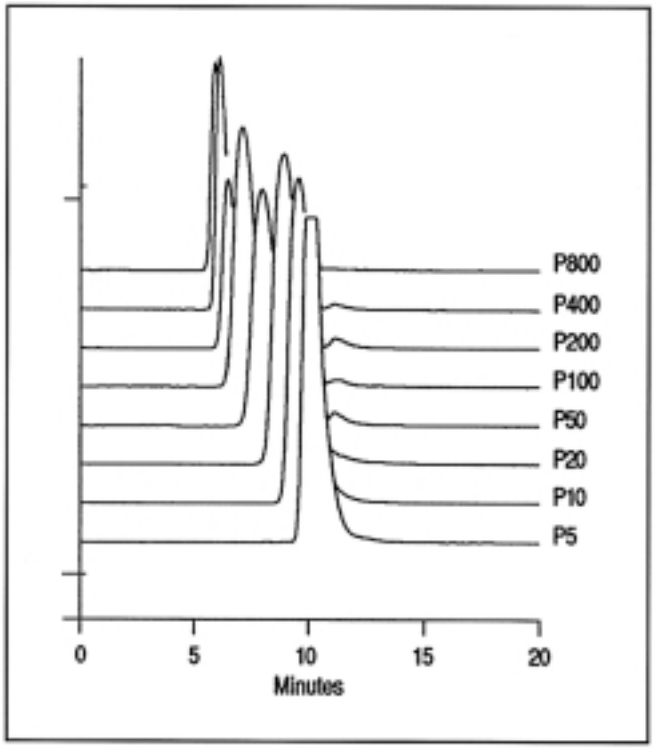

Figure 2. Size-exclusion chromatographic profile of pullulan fractions on the SE-450 column.

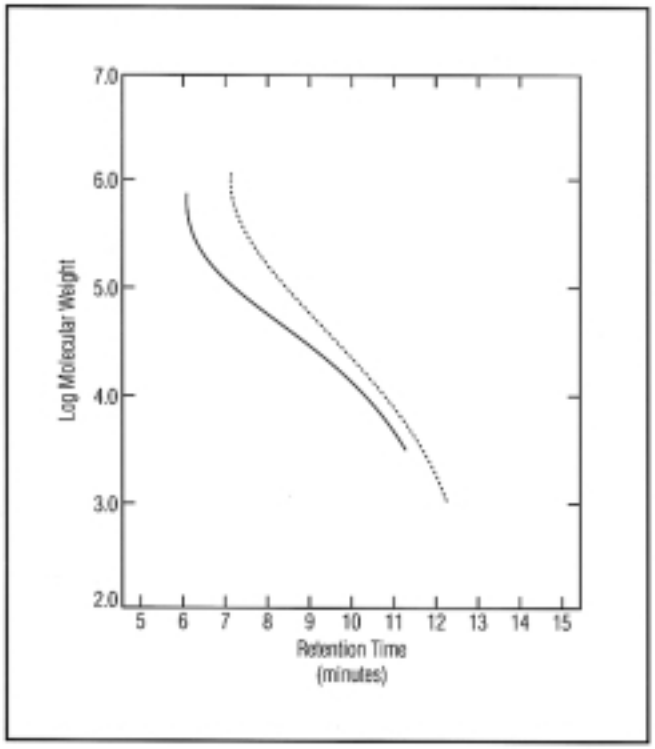

Figure 3. Pullulan standards on Zorbax SE-250 column. Dashed curve of globular proteins is referenced from Dionex product information bulletins.[10]

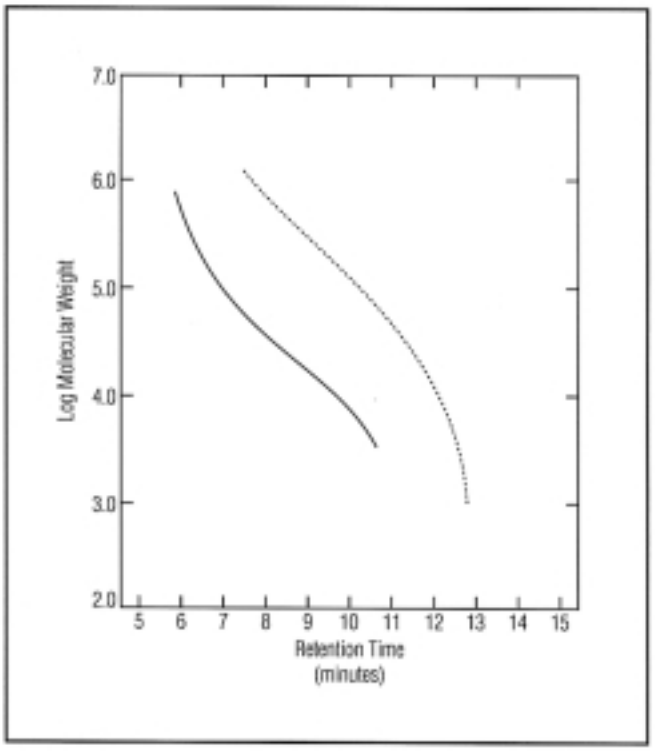

Figure 4. Pullulan standards on Zorbax SE-450 column. Dashed curve of globular proteins is referenced from Dionex product information bulletin.[10]

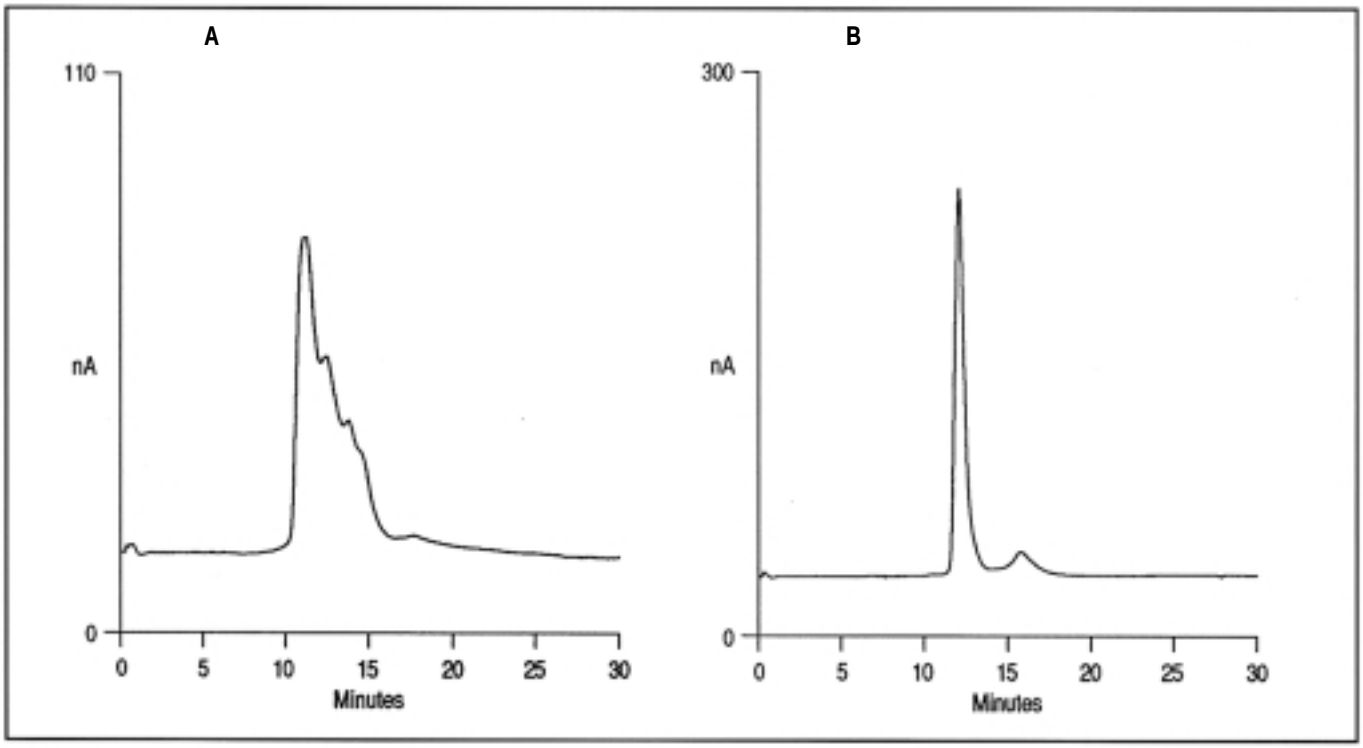

Figure 5. Gum ghatti on the SE-250 (A) exhibits a heterogeneous population of low molecular weight peaks. The same compound on the SE-450 (B) exhibits a major low molecular weight peak and a minor lower molecular weight peak that may be due to fragments.

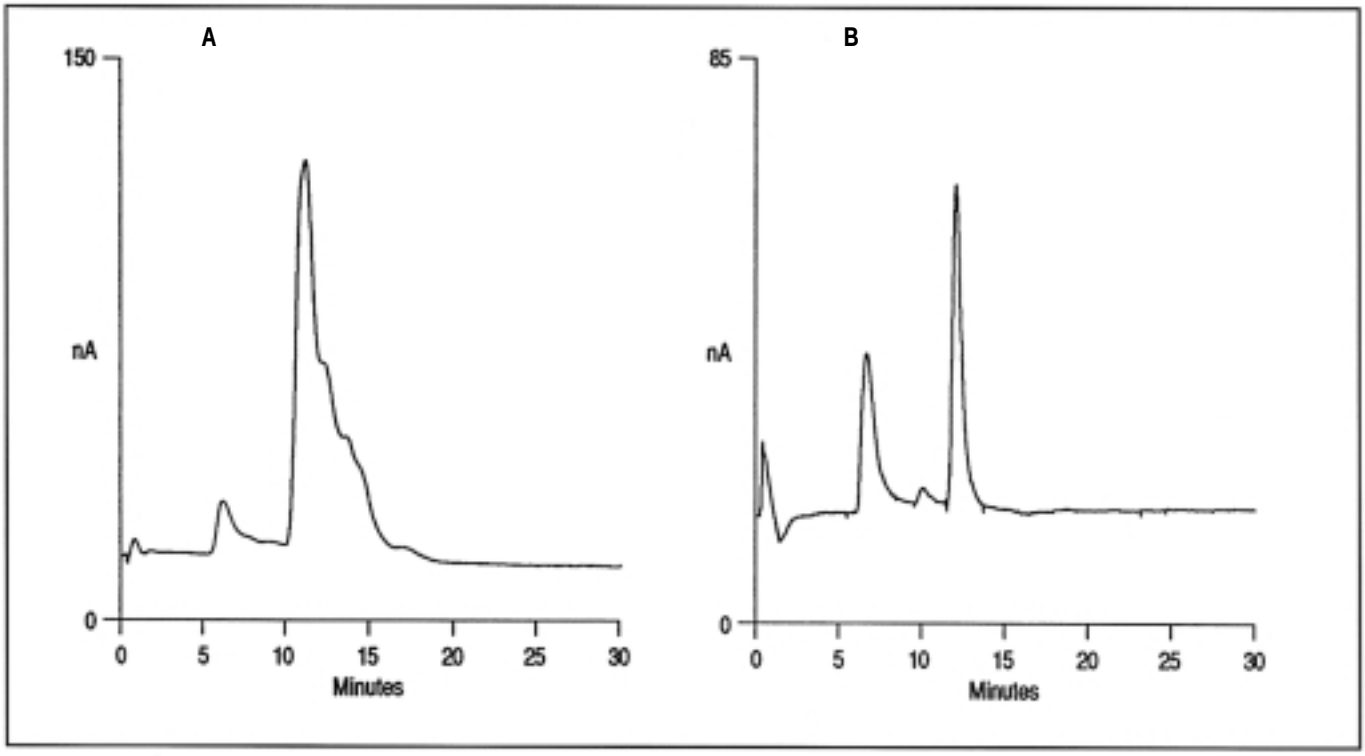

Figure 6. Locust bean gum on the SE-250 (A) exhibits a heterogeneous population with an additional peak excluded from the column. The same compound on the SE-450 (B) shows good resolution of some low molecular weight fractions, but others coelute, while the high molecular weight peak is still excluded.

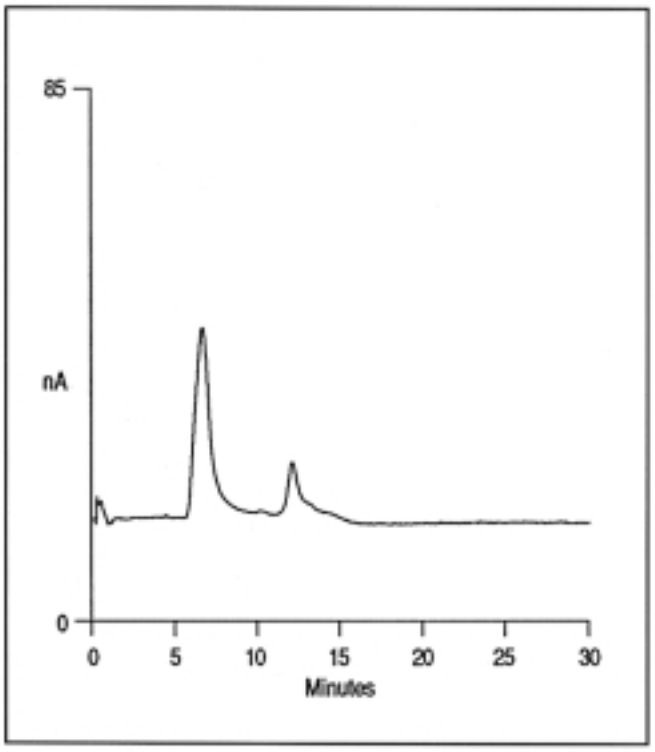

Figure 7. Analysis of chitosan on the SE-450 gives two distinct peaks, one low molecular weight, the other high.

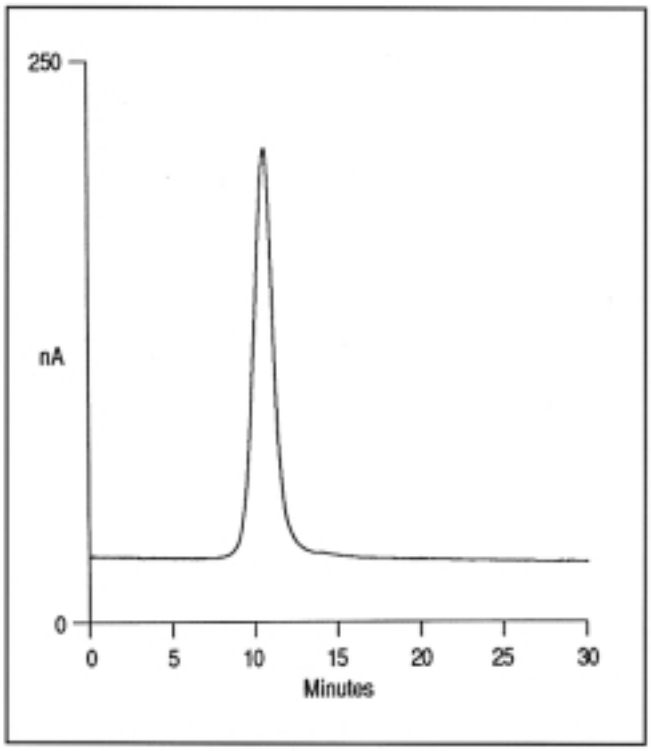

Figure 8. Analysis of dextran on the SE-450 shows a narrow distribution of peaks.

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