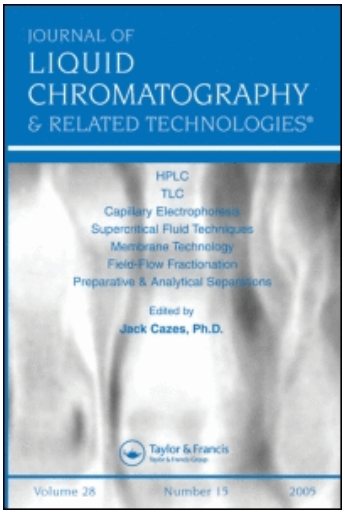

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High-Performance Gel Permeation Chromatography of Pectins

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HIGH-PERFORMANCE GEL PERMEATION CHROMATOGRAPHY OF PECTINS

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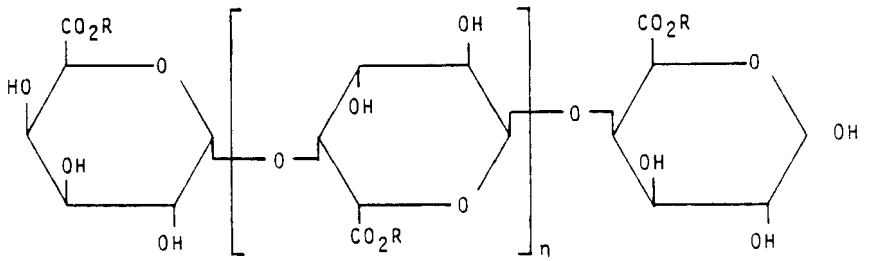
ABSTRACT

A high-performance gel permeation chromatographic (GPC) method was developed to determine the molecular weight distribution of pectins. The chromatographic system consisted of a hydrophilic coated silica (SynChropak) as the packing and a pH 3.7 acetate buffer as the mobile phase. By use of this system, high-methoxy, low-methoxy, and amidated pectins could be analyzed within fifteen minutes. By determining partition coefficients (K_d) of pectins as a function of mobile phase composition, K_d values were found to be independent of ionic strength from 0.055 to 0.7 M using pH 3.7 acetate buffers, which was in agreement with intrinsic viscosity data.

INTRODUCTION

Pectin is a natural polysaccharide isolated from the peel of citrus fruits. Purified pectin consists mainly of a linear polymer of α-1,4-linked D-galacturonic acid partially esterfied with methanol at the carboxyl on position C-6 (see Figure 1). Depending on the plant source and conditions used in its isolation, pectin usually contains varying amounts of D-galactose, L-arabinose, L-rhamnose and acetyl groups on C-2 and C-3 (1,2).

Pectin is used primarily in the food industry as a gelling agent for jams, jellies, and other foods and as a suspension stabilizer for milk products and frozen desserts. Pectin is also used in the pharmaceutical and cosmetic industries.



PECTIN D-GALACTURONAN

R = -H or $-CH_3$

FIGURE 1

Structure of pectin.

The gelation properties of pectin are strongly influenced by the polymer's degree of methylation (DM). Thus, pectins are classified according to DM values: high-methoxy pectins (DM, 60-80%) and low-methoxy pectins (DM, 25-50%) (3). Pectins can be hydrolyzed with ammonia to yield amidated low-methoxy pectins. Depending on DM values, pectins will gel in the presence of sugar and acid (high-methoxy) or Ca^{+2} (low-methoxy).

Although average molecular weights or degrees of polymerization of pectins are determined routinely from viscosity measurements (4,5), characterizing these polymers in terms of their molecular weight distribution (MWD) is highly useful in production control and end-use performance evaluation. However, there have been very few papers published concerning the MWD of this polysaccharide. Sepharose (6) and Sephadex (6-9) gels have been employed with dilute sodium chloride and/or sodium oxalate solutions as mobile phases.

During the past several years, high performance GPC supports for water-soluble polymers have been introduced. Because of their high efficiencies and short analysis times, these supports

are superior to conventional packings. Except for one brief report (10), the use of high-performance GPC for pectins has not been previously examined in detail.

This report describes the evaluation of a high-performance GPC system to determine relative MWD of high, low, and amidated methoxy pectins. This chromotographic system has been previously used to determine the MWD of modified cellulosics (11).

EXPERIMENTAL

Apparatus

A Varian 8500 liquid chromatograph and a Waters 401 differential refractometer were employed. The refractometer was thermostatted to 23-24°C with a Haake FE water bath. Stagnant mobile phase was kept in the reference side of the refractometer. Samples were injected with a Rheodyne 70-01 injection valve with a 20 µl loop.

Columns

The packing material consisted of a glycerylpropylsilyl layer covalently bonded to LiChrospher silica particles (10 µm) and was purchased prepacked in 4.1 mm I.D. x 25 cm long stainless steel columns from SynChrom, Linden, IN. Nominal pore sizes used in this study were 100, 500 and 1000Å. Columns were arranged in series with the smaller pore-sized support placed first.

Mobile Phase

All mobile phases were prepared using distilled water and reagent grade chemicals. They were filtered under vacuum using a 0.22 µm membrane filter (Type GS, Millipore, Bedford, MA). Ionic strengths were calculated using:

$$\mu = 1/2 \; \Sigma \; C_i Z_i^{\;2}$$

where C_i is the molar concentration of an ion of charge Z_i.

The degree of dissociation for acetate was estimated from the literature (12), at pH 4, $\alpha = 0.1$.

The recommended pH 3.7 mobile phase was prepared by first adding 60 ml of 4 M sodium acetate and 440 ml of 4 M acetic acid to a one-liter volumetric flask and filling to volume with water. This gives a pH 3.7 buffer of 0.22 M ionic strength. The ionic strength of this solution is then increased to 1.42 M by adding 0.4 mole of sodium sulfate to one liter of the 0.22 M acetate solution. This solution is then diluted 1:1 with water and used as the mobile phase. The 1.42 M solution is used for sample preparation.

Standards

Glucose was used to measure column efficiences and to determine the permeated column volume (V_p). Although a dextran 2×10^6 molecular weight standard could be used to determine the exclusion volume (Vo) of the 100 and 500Å columns, a calculated volume of 1.15 ml for each column was used in all K_d calculations. A list of all pectins mentioned in this study is given in Table 1.

Viscosity Determinations

Relative viscosities at 25°C were determined with a standard Ubbelohde capillary viscometer. Intrinsic viscosities were determined at 25°C with Ubbelohde capillary viscometers using at least six to eight concentrations and extrapolated to zero concentration. No shear rate corrections of the viscometers were made.

Recommended Procedure for the GPC Analysis of Pectins

Sample Preparation

A given amount (usually 1 mg/ml) of sample is slowly added to 50 ml of distilled water. To avoid agglomeration and lumping,

TABLE 1

Commercial Pectins Used in This Study

Type	Lot	DM[a]	DA[b]	AGA[c]	Mol. Wt.[d]
High-Methoxy	A	67%	---	82.4%	137,000
High-Methoxy	B	63%	---	82.6%	140,000
Low-Methoxy	A	34%	---	92.9%	58,000
Low-Methoxy	B	30%	---	88.6%	91,000
Amidated	A	27%	22%	88.8%	96,000
Amidated	B	26%	21%	89.3%	113,000

a) Degree of methylation

b) Degree of amidation

c) Anhydrogalacturonic acid

d) Determined from viscosity measurements

the sample is slowly sprinkled into the vortex of a rapidly stirred solution. Depending on the sample's viscosity, solutions must be stirred for a minimum of two hours and quite often overnight. After complete dissolution, 50 ml of 1.4 M ionic strength buffer (pH 3.7) is slowly added to a rapidly stirred solution. The solution is first prefiltered through a 0.7 µm glass-fiber filter (GF/F, 47 mm diam., Whatman) with subsequent filtration through a 0.65 µm membrane filter (Type DA, 13 mm diam., Millipore), using a Swinney holder. All samples must be chromatographed within two days after preparation.

When comparing peak profiles of different samples, each sample must be prepared in an identical fashion (length of stirring and filtration method).

Chromatographic Conditions

With a given column set and refractometer attenuation (x4 for 1 mg/ml solutions), 20 µl of sample solution is injected in

triplicate at a flow rate of 0.5 ml/min. To insure that chromatographic overloading effects are not occurring, it is recommended that several injections of lower concentration solutions be made and retention times and peak profiles compared.

RESULTS AND DISCUSSION

Effect of Mobile Phase Ionic Strength

The influence of mobile phase ionic strength in aqueous GPC has been covered in several reviews (12, 13), and recent papers (11, 13-19). Briefly, the ionic strength of the mobile phase must be sufficiently high to eliminate electrostatic interactions between ionic groups on the packing and on the polyelectrolyte. This will help prevent ion exclusion, ion inclusion, ion exchange, and adsorption from occurring. Not only does the addition of electrolytes in the mobile phase eliminate these non-size exclusion mechanisms, it can also reduce the polyelectrolyte's hydrodynamic volume. This decreases the viscosity of injected solutions, thus minimizing chromatographic viscosity effects. Moreover, analyzing polyelectrolytes in their contracted state may help to reduce the effect of chemical heterogeneity of ionic groups on the polymer.

To quantify the influence of mobile phase ionic strength on elution behavior of pectin, partition coefficients (K_d) were examined. These were calculated using the following equation:

$$K_d = \frac{Vr-Vo}{Vi}$$

where Vr is the elution volume of the sample as determined from its peak maximum, Vo is the total interstitial volume, which was obtained by assuming that it is equivalent to 35% of the total column volume, and Vi is the total pore volume, which was determined from the elution volume of glucose (V_p) using the following equation:

$$Vi = Vp-Vo$$

The influence of ionic strength on K_d was determined with a number of different pectin samples using a pH 3.7 acetate buffer and the results are shown in Table 2. As indicated, for a given

TABLE 2

Effect of Mobile Phase Ionic Strength on K_d[a] Values

Conditions: 100-500Å SynChrom columns (4.1 mm x 25 cm per column); mobile phase: pH 3.7 acetate buffer; flow-rate: 0.5 ml/min.; injection volume: 20 µl; detector: RI

Sample	Concentration mg/ml	K_d 0.055M	0.11M	0.41M	0.7M
High-Methoxy A	5.2	0.43			
	2.6	0.42	0.44	0.42	0.42
	1.0	0.40	0.41	0.41	0.39
High-Methoxy B	5.2	0.44			
	2.6	0.42	0.42	0.41	0.42
	1.0	0.41	0.41	0.42	0.41
Low-Methoxy A[d]	5.1	0.44			
	2.6	0.43	0.45	0.43[b]	0.42[b]
	1.0	0.41	0.42	0.41[c]	0.42[c]
Low-Methoxy B[d]	5.1	0.44			
	2.6	0.43	0.44	0.43[b]	0.42[b]
	1.0	0.41	0.42	0.42[c]	0.42[c]
Amidated A[d]	5.0	0.43			
	2.5	0.42	0.43	0.41[b]	0.41[b]
	1.0	0.40	0.41	0.39[c]	0.39[c]
Amidated B	5.2	0.43			
	2.6	0.42	0.43	0.42	0.42
	1.0	0.40	0.40	0.39	0.39

a) Calculated from peak maximum; Vp from glucose and Vo = 1.15 ml per column.
b) 2.1 mg/ml injected.
c) 0.8 mg/ml injected.
d) Samples contained ∿ 20% sucrose. Weights correspond to pectin and no moisture corrections were made.

concentration of injected polymer, there was no significant change in K_d values when the mobile phase ionic strength was increased from 0.055 to 0.7 M for all three types of pectin. When these results are compared with a similar study of carboxymethyl cellulose (CMC) an anionic polyelectrolyte, the pectins appear to be significantly less salt-sensitive than CMC in terms of K_d values (11) at higher ionic strength solutions.

The effect of ionic strength on the hydrodynamic volume of pectin was determined from intrinsic viscosity measurements of a high-methoxy pectin. As shown in Table 3, there was no significant intrinsic viscosity change when the ionic strength was increased from 0.05 to 0.7 M. This low salt sensitivity at higher ionic strength exhibited by pectin is in agreement with K_d measurements. The viscosity data are in agreement with results obtained by Lotzkar, et al. (20) in which the relative viscosities of 10.7 and 3.6% DM pectin samples reached minimum values at ionic strengths of about 0.01 and 0.09 M (pH $\sim$ 3), respectively.

As shown in Table 3, when the pH of the 0.7 ionic strength buffer was increased from 3.7 to 5.9, the intrinsic viscosity decreased from 3.4 to 3.2. This might have been caused by base-catalyzed depolymerization which apparently can occur at pH values greater than 4 (1).

TABLE 3

Effect of Ionic Strength on Intrinsic Viscosity of Pectin

Ionic Strength, M[a]	$[\eta]$[b]
0	15.0
0.05	3.5
0.10	3.4
0.35	3.4
0.70	3.4 (3.2 at pH 5.9)

(a) pH 3.7 acetate buffer

(b) HM-B pectin

Previously published intrinsic viscosities of CMC as a function of ionic strength at pH 3.7 showed that CMC is more salt-sensitive than pectin (11). This comparison is in agreement with results obtained by Pals and Hermans who studied the [η]-salt dependency of both pectin and CMC (21). Obviously, the salt sensitivity of the viscosity of these polyelectrolytes depends on the degree of substitution of ionic groups, the spatial configuration and flexibility of the polymer chain, and the compositional heterogeneity of ionic groups.

Effect of Mobile Phase pH

Pectin solutions are most stable at a pH range of 3-4. Outside this range, depolymerization can occur (1). Thus, our present studies were limited to a pH 3.7 buffer. However, Lotzkar has demonstrated that the relative viscosity of a 0.3% pectin solution was practically independent of pH (1.5 to 7.5) at an ionic strength of 0.017 M NaCl (20). At ionic strengths lower than 0.017, the relative viscosity of pectin increased with pH.

Recommended Mobile Phase

Based on GPC (Table 2) and intrinsic viscosity data (Table 3), the mobile phase ionic strength should be greater than 0.05 M to insure maximum polyelectrolyte contraction. A 0.7 M mobile phase is being routinely used in our laboratory. As previously described (11), at this high concentration small variations in mobile phase composition do not change peak shapes or K_d values. A pH 3.7 buffer has been selected to increase column life by reducing silica dissolution. At this pH, the mobile phase can be stored for extended periods of time because of the absence of microbiological growth. Moreover, this acetate-sulfate buffer is not corrosive. Solutions of pectin are stable at pH 3.7 (1) and soluble in the mobile phase providing that they are first dissolved in water as explained in the Experimental section.

Operational Parameters

1. Sample Size

As shown in Table 2 and Figure 2, the concentration of injected samples can have a significant effect on K_d values. As the sample concentration is increased, the elution volume of the peak increases. At higher concentrations, the peak becomes distorted. These overloading effects can be explained in terms of two phenomena (22): macromolecular crowding and viscous fingering. At high polymer concentration, it is assumed that

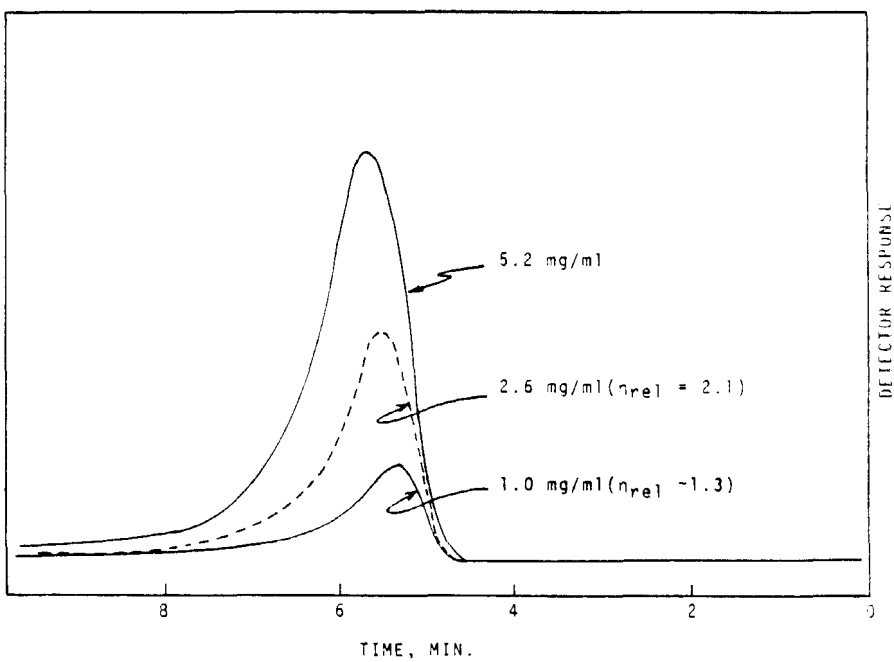


FIGURE 2

Effect of sample size on the elution profile of high-methoxy pectin lot B. Column set: 100-500Å SynChrom (4.1 mm I.D. x 25 cm long per column). Mobile phase: 0.55 μ (M) ph 3.7 acetate buffer. Flow-rate: 0.5 ml/min. Injection volume: 20 μl. Detector: RI x4

individual chains become crowded, thus reducing their hydrodynamic volume. This leads to a peak shift towards a higher elution volume. Peak distortion was first explained by Moore (23) in terms of a phenomenon called viscous fingering: if the viscosity of an injected solution is significantly greater than the mobile phase, the mobile phase will push through creating "fingers" of sample resulting in a distorted peak.

According to our findings, the relative viscosity of injected pectin solution must be less than 1.5 times the viscosity of the mobile phase to obtain K_d values which are independent of sample concentration (see Figure 2). These results are in agreement with a previous study of modified cellulosics (11). In order to achieve a relative viscosity of <1.5, the recommended pectin concentration is ~1 mg/ml using the acetate mobile phase.

Several possible methods of reducing viscosity effects include operating at high column temperatures or matching the viscosity of the mobile phase to the sample's viscosity. However, for 0.1% pectin solutions, relative viscosities was shown to be almost independent of temperature from 0 to 50° (24). Also, at high temperatures, there is the potential of decreasing column life.

Increasing mobile phase viscosity to match the viscosity of the sample will not only result in a greater pressure drop across the column, but also column efficiency will be decreased because of poor mass-transfer in the mobile phase. Another approach to avoid overloading effects is to inject larger volumes of more dilute solutions. However, as previously discussed (11), column efficiency is sacrified with larger injection volumes.

2. Column Selection

For most pectin samples, we have found 100-500Å or 100-1000Å column sets adequate. Additional columns can be used in series if higher resolution is required. Because of the presence of low molecular weight material in some samples and the occurrence of

a permeated peak of unknown origin in this chromatographic system (11), we suggest the use of a 100Å column to separate the totally permeated peak from the main polymer peak.

Characteristics of these columns, which include dextran calibration curves, HETP versus flow rate plots, and the influence of injection volume on column efficiency, have previously been published (11).

3. Column Calibration

Dextran can be used as a secondary standard to assign relative MWD to pectin samples. However, these assignments must be used on a relative basis for sample comparison. In the past, we have been using this GPC method to compare peak profiles among samples. More recently, we have been using an on-line, low-angle laser light-scattering detector for the measurement of absolute MWD (25).

4. Solution Preparation

Depending on the sample, insoluble protopectin (2) or aggregates (9, 26, 27) which contribute to the solution's haziness could be present in pectin solutions. This material can be removed by filtration (see Experimental section) and usually does not represent a significant portion of the sample. If significant amounts of insolubles are present, the danger of concentration polarization occurring on the membrane exists, and this might lead to ultrafiltration of the sample. Thus, the filtrate would not represent the soluble fraction of the sample. If ultrafiltration is suspected, sample pre-treatment by centrifugation or use of a larger pore filter with a large surface areas can be tried.

Applications

Figure 3 shows composite chromatograms of high-methoxy and low-methoxy samples using a 100-500Å column set. Although the molecular weights (determined from viscosity measurements) of the high-methoxy pectins were almost the same (Table 1), it can be

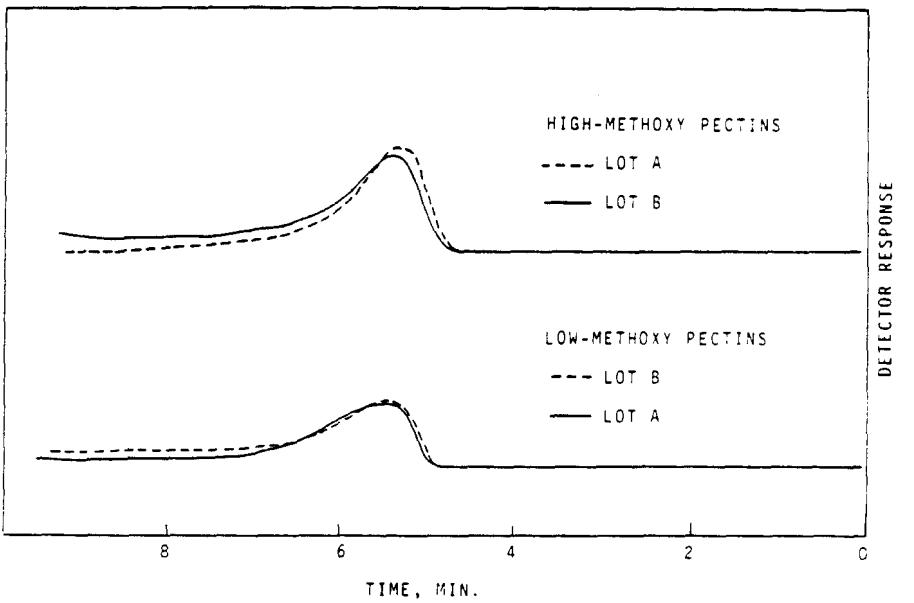


FIGURE 3

GPC analysis of pectins. Column set: 100-500Å SynChrom (4.1 mm I.D. x 25 cm longer per column). Mobile phase: 0.7 μ (M) pH 3.7 acetate buffer. Sample size: 1 mg/ml. Flow rate: 0.5 ml/min. Injection volume: 20 μl. Detector: RI x4

clearly seen that HM-A has more high molecular weight material and less low molecular weight material than HM-B. The LM-B sample contains more high and low molecular weight material than LM-A; from viscosity measurements LM-B is of higher molecular weight.

Figure 4 shows chromatograms of high-methoxy, low-methoxy, and amidated pectins using a 100-1000Å column set. Differences between the HM and LM samples with this column set are in agreement with those obtained on the 100-500Å column set. The amidated-B samples contains more high molecular weight material than sample amidated-A. This trend is in agreement with

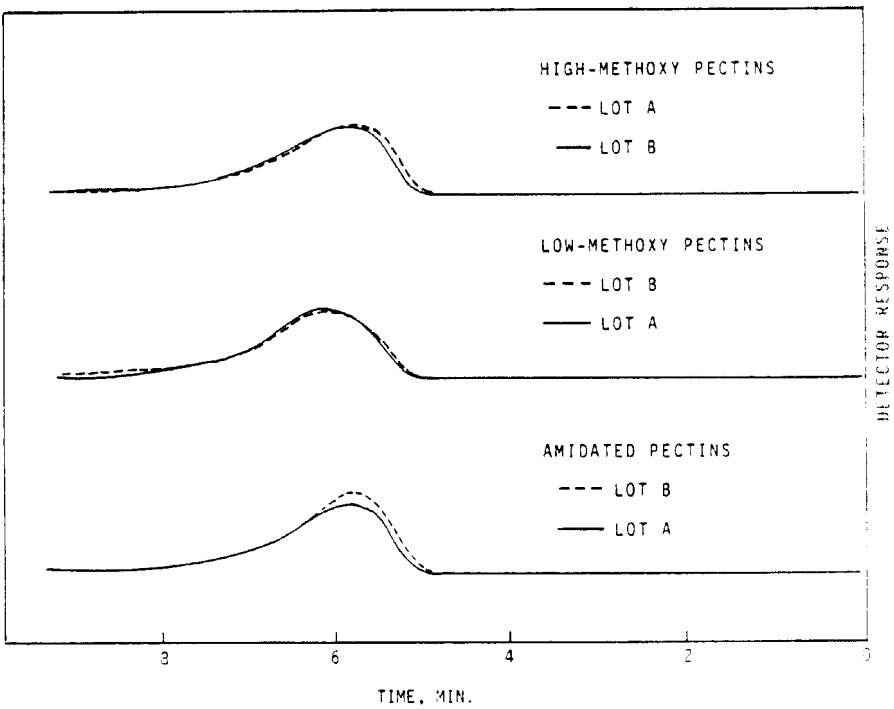


FIGURE 4

GPC analysis of pectins. Column set: 100-1000Å SynChrom. See Figure 3 for other conditions.

molecular weight measurements (Table 1). For clarity, the totally permeated peaks have been omitted from the chromatograms.

CONCLUSIONS

This study demonstrates that high performance GPC can be successfully used to compare the molecular weight distributions of high-methoxy, low-methoxy, and amidated pectins. By using hydrophilically modified silica particles as the GPC packing (SynChropak) and a high ionic strength buffer (0.7 M, pH 3.7), analyses were reproducible and no evidence of non-size exclusion mechanisms was observed.

To prevent chromatographic overloading effects, the relative viscosities of injected solutions must be below 1.5. Because of the high viscosity of pectins, low concentrations must be used (1 mg/ml).

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