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DETERMINATION OF MOLECULAR-WEIGHT DISTRIBUTION OF CHITOSAN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Optimal conditions for using high-performance liquid chromatography (HPLC) in the size exclusion mode have been determined for measuring the molecular-weight (MW) distribution of chitosan samples. Physical separation according to molecular size was accomplished on the stationary phase of glass supports having controlled pore sizes ranging from 2500 to 40 Å. Selection of column combinations was based on the requirements to resolve the higher MW fraction of chitosan and to give a linear calibration curve within the required MW range. The best combination of glass pore sizes and column lengths in two foot sections joined sequentially was: 2500 Å (2 ft.), 1500 Å (4 ft.), 550 Å (6 ft.), 250 Å (2 ft.), 100 Å (2 ft.), and 40 Å (2 ft.).

A loading study showed that an injection load of 500 μg, *i.e.* 100 μl at 5 g/l or 50 μl at 10 g/l (w/v), was the optimal load to give reproducible elution volumes, precision in quantitation, and minimum viscosity effects.

The best calibration curve using defined dextran standards was obtained from the geometric mean of $\bar{M}_w$ (weight average MW) and $\bar{M}_n$ (number average MW) values and peak elution volumes. Precision in determining MW distributions of chitosan as well as dextran standards was better than 5% relative standard deviation, and the differences between these results and the manufacturer's data on the dextran standards were 6 to −17%.

The MW distribution of a selected chitosan sample in 2% acetic acid thus determined was $\bar{M}_w = 2{,}055{,}000$, $\bar{M}_n = 936{,}000$, dispersity = 2.16, and the most abundant species was around 1,103,000. Analysis time for the HPLC separation was less than 20 min per sample.

Chitosan is an effective coagulating agent for the treatment of food processing wastes and activated sludge from biological treatment systems. It is manufactured from chitin in shrimp and crab wastes. The rapid methods developed here for determining the MW distribution of chitosan preparations will be used to optimize the manufacturing process and guide the selection of more effective chitosan products.

INTRODUCTION

Chitosan has been shown to be an effective coagulating agent for treatment of food processing waste effluents from vegetable, poultry, and egg breaking plants[1–3] and for conditioning of activated sludge produced from biological treatment of wastes[4]. Chitosan is the common name given to a class of carbohydrate polymers manufactured from chitin in shrimp and crab wastes. Protein and mineral components are extracted from these seafood processing wastes with dilute alkali and acid, respectively, leaving a chitinous residue. Chitin (poly-N-acetyl glucosamine) is converted to chitosan by deacetylation in hot concentrated alkali. This hydrolysis step removes some or all of the acetyl groups from chitin liberating amino groups which impart a polycationic nature to the chitosan product. The chitosan preparation consists of a mixture of different polymer sizes. The range of sizes or polydispersity of the molecular weight distribution is influenced by variables such as time, temperature, concentration, and atmospheric conditions employed in the deacetylation reaction. Thus chitosan products can have different characteristics of acetyl content, viscosity, molecular weight distribution, and performance as waste treatment agents.

The MW of a polymeric coagulating agent has been considered to be one of the most important characteristics affecting functionality of the polymer. It was shown that the optimal dosage of a synthetic cationic polymer, poly (diallyl dimethyl ammonium chloride), corresponded to the fraction with the optimal number average molecular weight[5].

The objective of this study was to develop a rapid method for determining the MW distribution of chitosan samples by high-performance liquid chromatography (HPLC). Porous glass supports coated with glycerol and having different controlled pore sizes were used to achieve the separation of molecules according to size.

EXPERIMENTAL

Materials

Dextran standards used (T-series) were obtained from Pharmacia (Piscataway, N.J., U.S.A.) and ranged in molecular weights from 10,000 (T-10) to 2,000,000 (T-2000) with defined weight average MWs ($\bar{M}_w$) and number average MWs ($\bar{M}_n$) obtained from light scattering and Sephadex gel filtration methods, respectively, for all except T-2000. Chitosan, batch 4-74, was supplied by Food, Chemical and Research Labs. (Seattle, Wash., U.S.A.). This is the same batch of chitosan employed in a previous study on coagulation of activated sludges produced by biological treatment on food processing wastes[4]. Chitosan was ground in a Wiley mill to pass a 20-mesh screen before use. Packing materials for HPLC columns were Corning's controlled pore glass supports having a covalently bonded glycerol coating (Glycophase-G/CPG) to inactivate free silicic acid groups on the surface of the glass particles. Glycophase-G/CPG supports were purchased from Pierce (Rockford, Ill., U.S.A.) except for the 2500 Å material which was supplied by Corning Glass (Medfield, Mass., U.S.A.). Pore sizes included 40, 100, 250, 550, 1500 and 2500 Å. The particle size of all the glass packing materials was 37–74 μm (200–400 mesh). Components purchased from Waters Assoc. (Milford, Mass., U.S.A.) included: an aqueous sample clarification kit, stainless steel columns (1/8 in. O.D. $\times$ 2 ft., with 5 μm sintered discs in

end caps), Series ALC/GPC-244 HPLC system with M 440 ultraviolet (UV) detector, R-401 refractive index (RI) detector, M 6000 A solvent delivery system, and U6K Septumless sample injector.

An OmniScribe recorder (Houston Instrument, Austin, Texas, U.S.A.) and an Autolab Minigrator (Spectra Physics, Santa Clara, Calif., U.S.A.) were used for recording and integrating the chromatograms. A compact computer, Compucorp Alpha 325 Scientist (Compucorp, Los Angeles, Calif., U.S.A.), was used for calculating MW distribution values.

Treatment of materials

All samples or standards were first dissolved in 2% reagent grade acetic acid and filtered through an aqueous sample clarification kit with MF-Millipore filter (pore size 0.45 μm). Chitosan samples were pre-filtered through a Gelman glass fiber filter (Type E) before passing through the Millipore filter kit .Glycophase-G/CPG was pretreated as follows: each bottle of porous glass (about 30 ml) was washed with 300 ml of tetrahydrofuran by shaking in a 500-ml erlenmeyer flask for 1 min, allowed to settle for 15 min and the supernatant decanted; 300 ml of distilled water was added, shaken and the water layer again decanted. The procedure was repeated a third time with another 300 ml of tetrahydrofuran. After decanting the supernatant, the slurry was dried in an oven overnight at 70°.

HPLC conditions

The manufacturers procedures for operating the HPLC were followed. Solvent flow-rate was 1 ml/min unless otherwise specified. Acetic acid (2%) was used as eluent in the solvent delivery system. The recorder speed was set at 1 cm/min. The Autolab Minigrator was in the "simulated distillation mode" in order to obtain peak heights at 10-sec intervals. Data from the RI detector were used to determine elution volumes and molecular weight distributions in this study. The UV detector was used to assist in measuring the separation of different chitosan components. A 254-nm filter was used in the UV light source with the UV sensitivity set at 0.05 a.u.f.s. The RI attenuation was set at 2× to 32× depending on the sample load with the preferred setting at 8× or greater to minimize signal noise. A 100-μl syringe was used for 50–100 μl injections; injection of other sizes were made with a 25-μl and 1-ml syringes. Sections of 0.009 in. I.D. tubing were used for connecting different columns. The most suitable injection size for dextran standards was determined to be 50 μl at 5 g/l. The optimal injection load of chitosan sample was studied by comparing the elution volume of the major chitosan component (peak) at various concentrations (0.625–30 g/l) and injection volumes. Columns were daily preconditioned by injecting 2 mg total load (*i.e.*, two injections of 100 μl at 10 g/l concentration) of chitosan in order to deactivate any residual active sites present in Glycophase-G/CPG.

Calibration of the molecular weight distribution

The calibration curves of dextran standards were plotted based on the peak elution volumes of standards *vs.* the logarithms of the weight average, ln $\bar{M}_w$; arithmetic average of $\bar{M}_w$ and $\bar{M}_n$, ln $([\bar{M}_w + \bar{M}_n]/2)$; or the geometric average (ln $\bar{M}_w$ + ln $\bar{M}_n$)/2 of each standard. An equation describing the linear region of the calibration curves is given as ln $(M_i) = a + b \cdot V_e$ (where M_i = MW of species i, V_e = elution

volume). The intercept value, a, and slope value, b, were calculated from the linear regression equation describing the best fit of the elution volumes of standards treated as unknowns. The MWs of the chitosan samples used in this study were assumed to be within the linear range of the calibration curve, because the 2500 Å column would extend the void volume to correspond to MW values of approximately 40 million[6,7]. A void volume marker in this range was not available. With the slope and intercept of the calibration curves, and with the heights taken at fixed 10-sec intervals by the Minigrater, the $\bar{M}_w$ and $\bar{M}_n$ of each sample could be calculated from different elution volumes.

The equations for calculating $\bar{M}_w$ and $\bar{M}_n$ are as follows:

Since $h_i = k \cdot W_i = k \cdot N_i \cdot M_i$

$$\bar{M}_n = \frac{\Sigma (N_i \cdot M_i)}{\Sigma N_i} = \frac{k \Sigma h_i}{k \Sigma (h_i/M_i)} = \frac{\Sigma h_i}{\Sigma (h_i/M_i)}$$

$$\bar{M}_w = \frac{\Sigma (N_i \cdot M_i^2)}{\Sigma (N_i \cdot M_i)} = \frac{k \Sigma (h_i \cdot M_i)}{k \Sigma h_i} = \frac{\Sigma (h_i \cdot M_i)}{\Sigma h_i}$$

thus

$$\bar{M}_w = \frac{\Sigma (h_i \cdot M_i)}{\Sigma h_i}, \quad \bar{M}_n = \frac{\Sigma h_i}{\Sigma (h_i/M_i)}$$

where $M_i = \exp(a + b \cdot V_e)$, h_i is the height at each 10-sec interval of the chromatogram peak, W_i represents the total weight of that species, k the constant of the signal and represents the relationship between the signal height and actual weight, and N_i the number of molecules.

In using the calibration curve to estimate chitosan MWs based on the dextran standards, MW–MW correlations were used rather than size–size correlations, because the calibration factors (Q, MW units per Å) for the dextran standards as well as chitosan were not available. It was assumed that the calibration factors for dextran standards and chitosan were approximately equal because of their similarity in structure and the difference between them was within the range of precision obtainable by HPLC determination of MW distribution.

RESULTS AND DISCUSSION

Selection of optimal column combinations

As indicated by preliminary studies, the sample of chitosan under examination had an MW ranging from the region of T-10 (10,000) to greater than T-2000 (2,000,000). Thus, a calibration curve having linearity in this range was required. Since molecular size exclusion was the separation mode used in this study, the column length and pore sizes of the packing materials were the primary factors to be manipulated. The efficiency of different column combinations was studied in terms of the linearity of dextran standard curves as well as the separation of different MW components in the chitosan sample. From the study shown in Fig. 1 some relationships between the dextran stan-

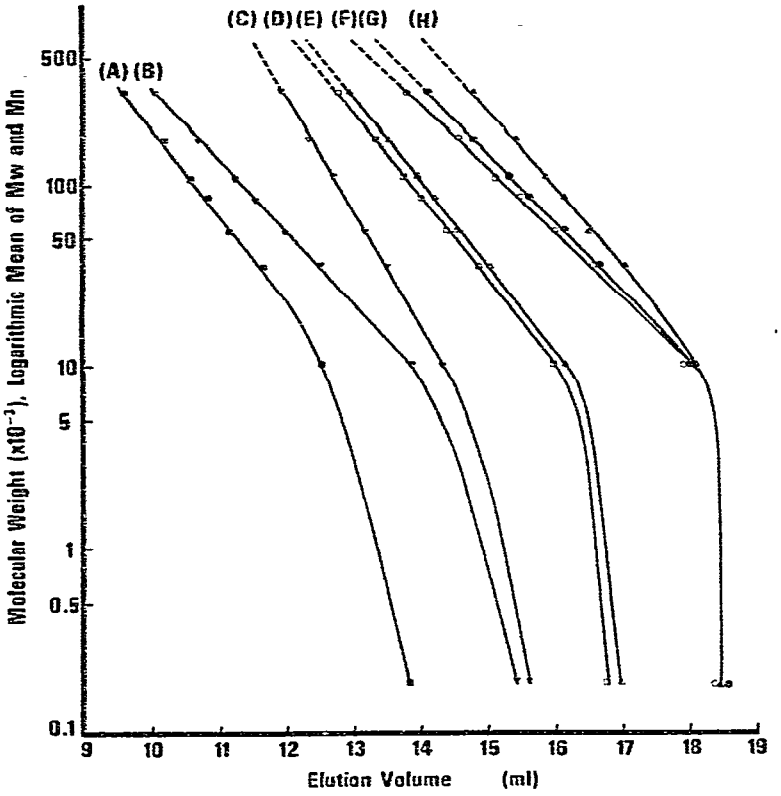

Fig. 1. Calibration curves based on the geometric averages of $\bar{M}_w$ and $\bar{M}_n$ values of dextran standards *vs.* their peak HPLC elution volumes with different column combinations; (A) 0–4–4–2–2–0, (B) 0–4–4–2–2–2, (C) 4–2–2–2–2–2, (D) 2–4–4–2–2–2, (E) 4–2–4–2–2–2, (F) 2–4–6–2–2–2, (G) 0–6–6–2–2–2, and (H) 4–4–4–2–2–2. Code numbers represent length of columns (ft.) in the order of 2500, 1500, 550, 250, 100, and 40 Å. Other conditions included: solvent, 2% acetic acid; columns, 1/8 in. O.D.; flow-rate, 1 ml/min; temperature, ambient; standards, dextran T-10, T-40, T-70, T-150, T-250, and T-500, and glucosamine (5 g/l).

dards and pore sizes were obtained. First, by adding a 2-ft. length of column containing the 40-Å glass packing material, the linear region of the curve was extended from a MW value of 30,000 (curve A) to 10,000 (curve B) and the slope was decreased. Second, the presence of larger pore size materials (curves C–H) led to greater elution volumes, increased the void volume, and extended linearity toward higher MW values which was desirable in this study. The slope of curve F was less than for other curves in the series C–H. Thus, this combination of column lengths (ft.), 2–4–6–2–2–2, of 2500, 1500, 550, 250, 100, and 40 Å porous glass supports gave the broadest working range for separation of different MW components of chitosan.

The separation of chitosan in 2% acetic acid was also studied under different column combinations. The results are shown in Fig. 2 as line drawings indicating the position of peak elution volumes of usual chitosan components. Chitosan could be separated into three regions with three peaks, P-I, P-II, and P-III (Fig. 3). The one eluting first, P-I, had an approximate MW of 10 million; its amount was small com-

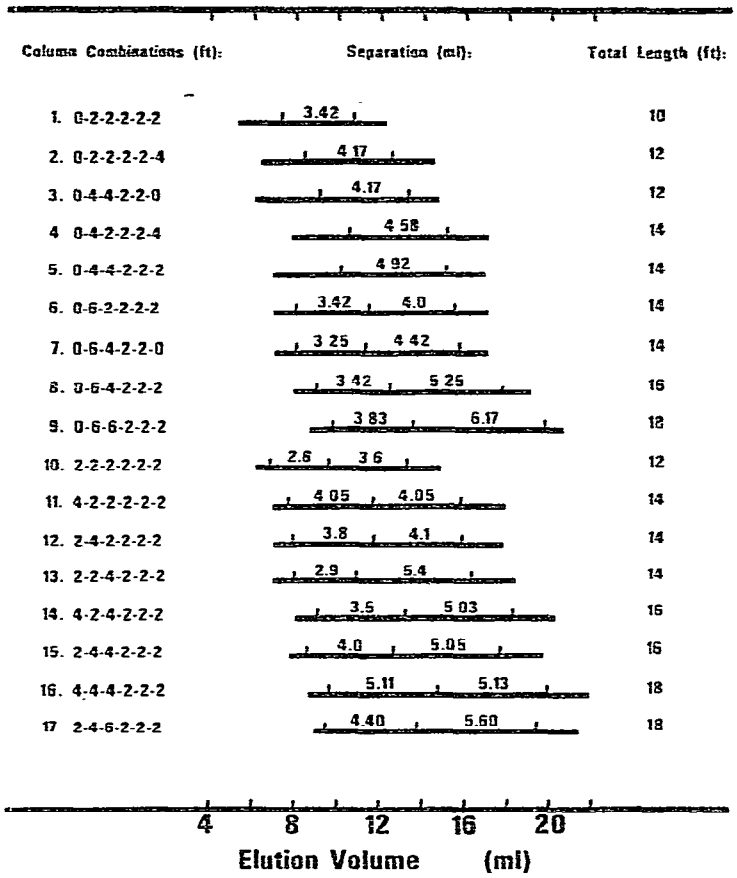

Fig. 2. Comparison of the efficiency of HPLC with different column combinations for separating different MW components in a chitosan solution. Column lengths were coded as indicated in Fig. 1 and each line represents the location on the HPLC chromatograms of RI peaks and the separation between peaks is indicated (ml).

pared with other fractions, but it had a very strong UV (254 nm) absorption. This UV peak was used, in addition to the RI peaks, for comparing the separation of P-I and P-II. Approximately 90% of the total area under the curve was contained in the P-II fraction. A negative peak, P-III, having a refractive index less than that of the 2% acetic acid solvent, eluted in the region of total elution volume (V_t). Since fractions around P-I and the heavier side of the P-II regions (with MW greater than 1,100,000 as shown later) were considered to be the main components that affected the viscosities and perhaps the coagulation effectiveness of different products, high resolution in this region and better separation of P-III fraction from this region were the primary criteria for selecting the optimum column combination. Components in the P-III region were considered to have MWs less than 10,000 and were not significant in this study. Without the 2500-Å column and with 2–4 ft. of 1500-Å column as shown in lines 1–5 of Fig. 2, P-I was mixed with P-II. Adding the 2500-Å column, using 6 ft. of 1500-Å column, or various combinations of 1500- and 2500-Å columns gave sepa-

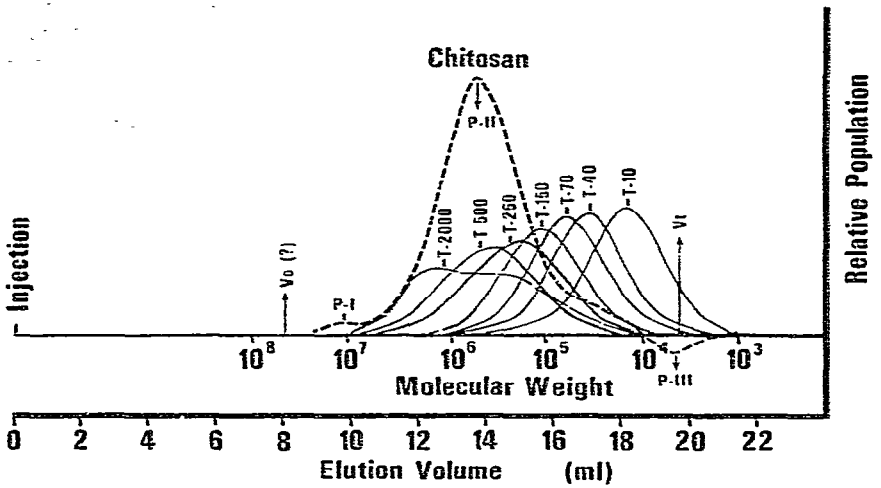

Fig. 3. Refractive index response curves illustrating elution patterns and MW distributions of dextran standards and chitosan fractionated by HPLC with column combination F (2–4–6–2–2–2).

ration of P-I and P-II as shown in lines 6–17. When the 2500-Å or the 1500-Å columns were lengthened, P-I and P-II were separated better than when the 550-Å column was lengthened (lines 11, 12 and 13; or lines 14 and 15). The 550-Å column showed a beneficial effect on separating P-II and P-III (observed among lines 11 to 13, or between 16 and 17). It was concluded that the 2500-Å and the 1500-Å columns were efficient in separating the fraction with MW larger than P-II (which was about 1,100,000 after

TABLE I

EFFECT OF CHITOSAN LOAD ON COLUMN EFFICIENCY (N)

Experimental conditions are described in the text. V_e = elution volume (peak II is the major peak of chitosan); $N = 16\ (V_e/\text{width})^2$; nm = peak is too small to be measured.

Total load (μg)	*Injection size (μl)*	*Concentration (g/l)*	*RI sensitivity*	*Peak II*		
				V_e (ml)	*Width (ml)*	*N*
Fixed injection size (different concentrations)						
1000	100	10.0	16×	11.85	4.0	140.4
750	100	7.5	16×	11.55	3.8	147.8
500	100	5.0	16×	11.30	3.6	157.6
250	100	2.5	8×	10.75	3.5	150.9
125	100	1.25	4×	10.65	3.2	177.2
62.5	100	0.625	2×	10.55	3.2	173.9
Fixed concentration (different injection sizes)						
⩾2000	⩾200	10.0	32×	(overloaded)		
1000	100	10.0	16×	11.70	3.7	160.0
500	50	10.0	8×	11.20	3.5	163.8
250	25	10.0	8×	10.75	3.2	180.6
125	12.5	10.0	4×	10.60	2.6	256.9
100	10	10.0	4×	10.60	2.3	339.8
50	5	10.0	2×	10.60	nm	—

calibration with dextran standards later in this study). The columns with pore sizes of 550 Å and smaller were effective in separating fractions between P-II and P-III (which were below 1,100,000). Therefore, the combination 2–4–6–2–2–2 (F, Fig. 1) was considered as optimal for characterization of chitosan components. The total column length was 18 ft. which created a back pressure of around 3000 p.s.i. at 1 ml/min flow-rate. The chromatograms (RI response curves) of dextran standards and chitosan obtained with this optimal column combination are shown in Fig. 3. The shape of the curves for Dextrans T-10, 40, 70, 150, 250, and 500 approximates gaussian distributions. The curve obtained for T-2000 clearly shows it contains a wide distribution of MW components and no one predominant species. The polydispersity of each standard as well as chitosan was shown in the figure. No corrections for band broadening due to diffusion have been made in this study.

Loading study

A loading study was conducted by varying the concentration or the injection size (Table I), or both but with the total load fixed (Table II). Extremely viscous solutions would be expected to cause band broadening and higher elution volumes during HPLC due to drag forces along the inner walls of the 1/8-in. columns. Viscosity effects were considered to be eliminated when elution volumes of the peaks became constant as was observed at a concentration of 1.25-g/l and 100-μl injections or with

TABLE II

EFFECT OF CHITOSAN CONCENTRATION AND INJECTION SIZE ON COLUMN EFFICIENCY (N)

Experimental conditions are described in the text. $N = 16\,(V_e/\text{width})^2$. Measurements 1–14 (October 30th, 1975) are single runs, 15–19 (October 31st, 1975) the results of 4 replications.

Measurement No.	*Total load (μg)*	*Concentration (g/l)*	*Injection size (μl)*	*Peak II*		
				V_e (ml)	*Width (ml)*	*N*
1	250	0.625	400	10.85	3.4	—
2	250	1.25	200	10.85	3.6	—
3	250	2.5	100	10.85	3.6	145.3
4	250	5.0	50	10.80	3.4	161.4
5	250	10.0	25	10.85	3.6	149.5
6	250	20.0	12.5	11.05	3.7	142.7
7	250	30.0	8.33	11.15	3.8	137.8
8	500	0.625	800	11.25	3.9	—
9	500	1.25	400	11.15	3.9	—
10	500	2.5	200	11.10	3.6	152.1
11	500	5.0	100	11.15	3.6	153.5
12	500	10.0	50	11.10	3.7	144.0
13	500	20.0	25	11.25	3.8	140.2
14	500	30.0	16.7	12.30	4.0	151.3
15	250	5.0	50	11.00	3.8	134.1
16	250	10.0	25	11.01	3.9	127.5
17	500	5.0	100	11.58	4.2	121.6
18	500	10.0	50	11.63	4.25	119.8
19	1000	10.0	100	11.99	4.58	109.7

10-g/l and 12.5-μl injection. These results suggested a total load of 125 μg should be used. Tables I and II also show that the efficiency, N, increased as the total load decreased. However, the sensitivity settings of 4 or 2× required for detection at loadings below 250 μg resulted in excessive noise in the detector output. A smooth strong signal was desired for measurement of signal heights and calculation of MW distribution. A stronger signal was obtained by injecting a sample load of 500 μg than when a 250-μg load was used.

At total loadings of 250 and 500 μg (Table II), viscosity effects were observed to result in increased elution volumes and peak widths when the concentration exceeded 10 g/l. At a total load of 500 μg, the injections with concentrations of 10 g/l or less gave constant elution volumes even though the viscosity effect was present as indicated before. These results suggested that there was a marginal viscosity effect with injections of 100 μl with 5 g/l or 50 μl with 10 g/l, yet, the results were reproducible; furthermore, this total load gave a stronger RI signal with less system noise providing a more precise quantitation of the MW distribution than the total load of 250 μg. Therefore, the optimal total load was considered to be 500 μg at the concentrations of 5 or 10 g/l.

Calibration curves and calculation of molecular-weight distribution

The dextran standards obtained for this study were not homogeneous samples. Each of these standards contained molecules of a range of MWs with defined $\bar{M}_w$

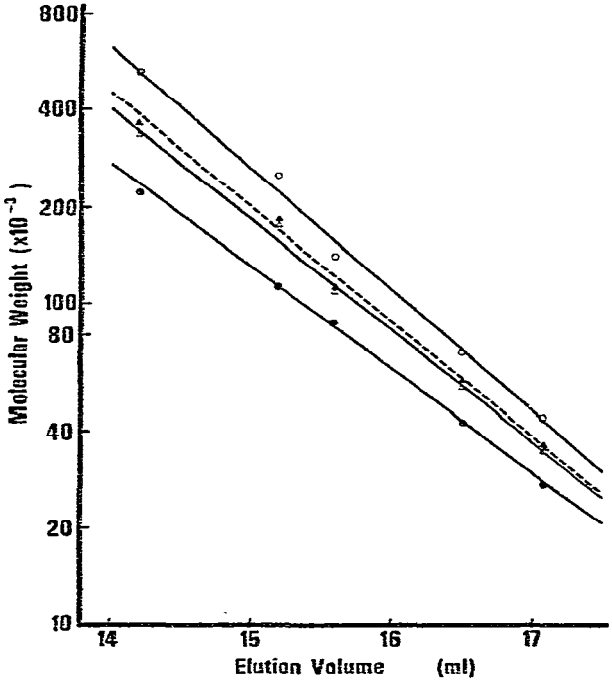

Fig. 4. Calibration curves based on the elution volumes at RI peaks of dextran standards eluted by HPLC with column combination F (Fig. 1) *vs.* values of (1) ln $\bar{M}_w$ (○), (2) ln $[(\bar{M}_w + \bar{M}_n)/2]$ (▲), (3) $(\ln \bar{M}_w + \ln \bar{M}_n)/2$ (△), or (4) ln $\bar{M}_n$ (●), obtained from $\bar{M}_w$ and $\bar{M}_n$ values of dextran standards given by Pharmacia.

and $\bar{M}_n$ values given by Pharmacia. Thus, several calibration curves could be plotted differently from these standards as shown in Fig. 4 based on elution volumes of peaks and their various MW values. In order to choose the best calibration curve, four calibration curves: (1) V_e *vs.* $\ln \bar{M}_w$, (2) V_e *vs.* $\ln [(\bar{M}_w + \bar{M}_n)/2]$, the logarithm of arithmetic mean, (3) V_e *vs.* $(\ln \bar{M}_w + \ln \bar{M}_n)/2$, the geometric mean, and (4) V_e *vs.* $\ln \bar{M}_n$, were fitted with linear regression, and used for calculating $\bar{M}_w$ and $\bar{M}_n$ values of several dextran standards considered as if they were unknowns. Among these curves, the curve in the higher position in Fig. 4 tended to give higher estimations of MWs than the lower curves. Theoretically, the curve (open triangles in Fig. 4) based on the geometric means should give the closest estimations to the defined $\bar{M}_w$ and $\bar{M}_n$ values since $\bar{M}_w$ and $\bar{M}_n$ fall on either side of the crest of a chromatogram peak with a gaussian distribution so that the elution volume at the peak should correspond to the geometric mean of its $\bar{M}_w$ and $\bar{M}_n$ on the semilogarithmic plot better than other parameters. Dextran T-2000 was not used in the calibration study because of its non-gaussian dispersity as shown by results in Fig. 3 (and Table III).

In Fig. 5, the estimated $\bar{M}_w$ and $\bar{M}_n$ values (with open circles) based on the $\ln \bar{M}_w$ curve in Fig. 4 were all much higher than Pharmacia's values shown as a solid line for their $\bar{M}_w$ values and as a dashed line for their $\bar{M}_n$ values. The estimated $\bar{M}_w$ and $\bar{M}_n$ values based on the $\ln \bar{M}_n$ curve in Fig. 4 were not shown in Fig. 5, because they were all below Pharmacia's values and kept out of the figure for the sake of clarity. The $\bar{M}_w$ and $\bar{M}_n$ values estimated by the calibration curve (Fig. 4) based upon geometric means, shown as open triangles in Fig. 5, were observed to be closest to the

TABLE III

MOLECULAR WEIGHT DISTRIBUTION OF DEXTRAN STANDARDS DETERMINED BY HPLC

$\bar{M}_{g\ avg}$ is the geometric average MW: $\exp [(\ln \bar{M}_w + \ln \bar{M}_n)/2]$. $\Delta\bar{M}_w$, $\Delta\bar{M}_n$, ΔPeak = % deviations from Pharmacia's data. Data in parentheses are given by Pharmacia. Determinations based on the standard curve fitted by linear regression as: $\ln M_t = a + b \cdot V_e$, where $a = 23.9215$, $b = -0.0131$, V_e is in one sixtieth of a ml (or seconds under 1 ml/min flow rate), and M_t is the $\bar{M}_{g\ avg}$ of each standard. D (dispersity) $= \bar{M}_w/\bar{M}_n$.

Dextran	$\bar{M}_w \times 10^{-3}$	$\bar{M}_n \times 10^{-3}$	D	$\bar{M}_{g\ avg} \times 10^{-3}$	*Peak MW* $\times 10^{-3}$	$\Delta\bar{M}_w$ (%)	$\Delta\bar{M}_n$ (%)	Δ*Peak MW* (%)
T-2000	—	—	—	—	(2000)*			
	832.1	184.4	4.51	—	1335*	—	—	—
T-500	(516)	(212)	(2.43)	(330.7)	(330.7)			
	482.8	199.4	2.42	310.3	350.9	−6.4	−5.9	6.1
T-250	(253)	(112)	(2.26)	(168.7)	(168.7)			
	224.5	108.1	2.08	155.8	156.8	−11.3	−3.5	−7.1
T-150	(141)	(86)	(1.64)	(110.2)	(110.2)			
	131.2	71.3	1.84	96.7	116.8	−7.1	−17.1	6.0
T-70	(70.0)	(42.5)	(1.65)	(54.6)	(54.6)			
	69.2	40.6	1.70	53.0	57.1	−1.1	−4.5	4.6
T-40	(44.9)	(27.8)	(1.62)	(35.3)	(35.3)			
	39.7	24.9	1.59	31.4	−6.2	−12.2	−10.4	2.5

* The chromatogram of T-2000 was not a normal distribution curve; thus, the highest point of the curve was taken as the peak.

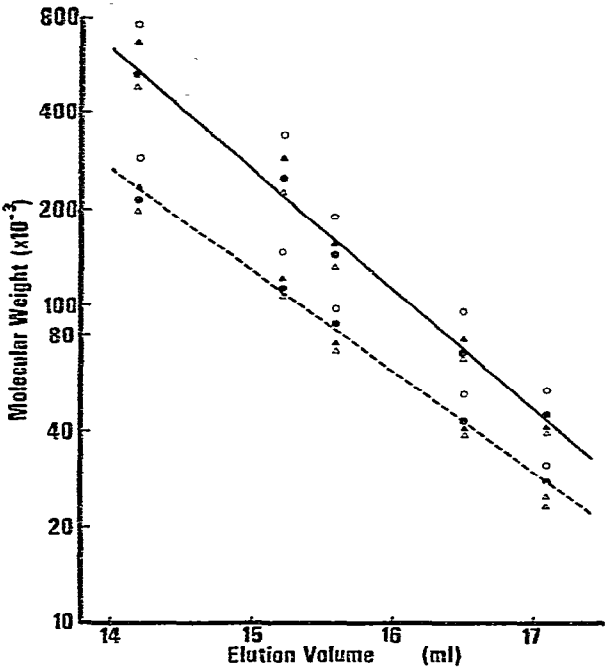

Fig. 5. Comparison of $\bar{M}_w$ and $\bar{M}_n$ values of five dextran standards calculated from: (1) the calibration curve (no. 1, Fig. 4) based on the logarithm of $\bar{M}_w$ *vs.* V_e (○), (2) the calibration curve (no. 2, Fig. 4) based on the logarithm of arithmetic average of $\bar{M}_w$ and $\bar{M}_n$ *vs.* V_e (▲), and (3) the calibration curve (no. 3, Fig. 4) based on the geometric average of $\bar{M}_w$ and $\bar{M}_n$ *vs.* V_e (△), and compared to the $\bar{M}_w$ (●—●) and $\bar{M}_n$ (●—●) values of these standards given by Pharmacia.

values given by Pharmacia. The $\bar{M}_w$ and $\bar{M}_n$ values calculated from this calibration curve (open triangles) are compared mathematically with Pharmacia's data in Table III. Both $\bar{M}_w$ and $\bar{M}_n$ values were about 10% different from Pharmacia's data, except $\bar{M}_n$ for T-150 which was 17% lower. All peak MWs were within 10% of Pharmacia's data. The same type of mathematical comparison was also done on the estimation based on the arithmetic mean and it showed the deviations were greater than those from the geometric mean. One should notice from Figs. 4 and 5 that a slight difference in the calibration curve (such as with arithmetic or geometric means) would bring about a pronounced difference in the prediction of $\bar{M}_w$ and $\bar{M}_n$. When the curve in Fig. 4 which was based on arithmetic means was used as the calibration curve, $\bar{M}_w$ and $\bar{M}_n$ values were overestimated. When the curve which was based on the geometric means was used as the calibration curve, $\bar{M}_w$ and $\bar{M}_n$ values were underestimated, yet closer to Pharmacia's data. One can even improve the estimation by seeking a calibration curve falling between lines of the arithmetic and the geometric mean (by doing this, less than 5% deviation from Pharmacia's data has been achieved). However, for practical purposes, the geometric mean of $\bar{M}_w$ and $\bar{M}_n$ is a reproducible parameter and gives enough accuracy, especially when the reproducibility of the experiment shows acceptable precision (Table IV).

Using the calibration curve based upon the geometric mean of $\bar{M}_w$ and $\bar{M}_n$ values of seven dextran standards *vs.* their peak elution volumes (Fig. 4), the MW

TABLE IV

REPRODUCIBILITY CF HPLC IN DETERMINING THE MOLECULAR-WEIGHT DISTRIBUTIONS

D (dispersity) = $\bar{M}_w/\bar{M}_n$; S.D. = standard deviation; % error = relative standard deviation.

Sample	*Parameter*	*Peak MW* $\times 10^{-3}$	$\bar{M}_w \times 10^{-3}$	$\bar{M}_n \times 10^{-3}$	*D*
Chitosan (4-74)					
(4 replications)	Mean	1103	2055	936	2.16
	S.D.	22.2	27.7	23.3	0.031
	% Error	2.01	1.34	2.48	1.43
Dextran T-250					
(3 replications)	Mean	156.8	225.2	114.3	1.97
	S.D.	0.0	1.65	5.38	0.095
	% Error	0.0	0.73	4.70	4.82
Dextran T-40					
(3 replications)	Mean	36.3	46.0	25.5	1.81
	S.D.	0.3	0.57	0.69	0.05
	% Error	0.82	1.23	2.70	2.76

distribution of chitosan shown in Table IV was $\bar{M}_w$ 2,055,000, $\bar{M}_n$ 936,000, dispersity 2.16, and the MW of the highest peak corresponding to the most abundant species was 1,103,000. Since these data were based on the MW of dextrans, a calibration factor for dextran, Q factor[8], or the relationship between Q factors for dextran and chitosan is needed in order to obtain the true MW of chitosan. For this purpose, X-ray diffraction and ultracentrifugation studies are being conducted. However, for the purpose of comparing different chitosan samples, the calculation without the correction for the difference in Q factors was assumed to be adequate.

Reproducibility

As shown in Table IV, the precision (% errors) for estimating the peak MW, $\bar{M}_w$, $\bar{M}_n$, and dispersity of the chitosan solution were 2.01, 1.34, 2.48, and 1.43, respectively. The V_e at peak of the chromatogram obtained for dextran T-250 was repeatably at 15.2 ml which corresponded to a peak MW of 156,800. Percent errors for estimating the peak MW, $\bar{M}_w$, $\bar{M}_n$, and dispersity of dextran T-250 were 0.0, 0.73, 4.70, and 4.82, respectively. The V_e of dextran T-40 was at 16.8 ml which corresponded to a peak MW of 36,300. Percent errors for estimating the peak MW, $\bar{M}_w$, $\bar{M}_n$, and dispersity of dextran T-40 were 0.82, 1.23, 2.70, and 2.76, respectively. The sources of error included (1) the base line drift of the RI detector, (2) the performance of the injector, the pump unit, and columns, (3) the precision in determining the calibration curve, (4) the injection size, and (5) the uniformity of the sample solution.

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