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EVALUATION OF ROOT-MEAN-SQUARE RADIUS OF GYRATION AS A PARAMETER FOR UNIVERSAL CALIBRATION OF POLYSACCHARIDES

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ABSTRACT

High-performance, size-exclusion chromatography columns were calibrated in average root-mean-square radii of gyration ($\bar{R}_{gz}$) by a combination of commercial "narrow" pullulan and "broad" dextran standards. The nonlinear calibration-curves were fitted by a computer-aided, iterative, least-squares procedure. Values of $\bar{R}_{gz}$, obtained from a point-by-point transformation of the respective pullulan and dextran chromatograms by utilizing universal calibration, were compared with inputted $\bar{R}_{gz}$ calibration values. For standards ranging in $\bar{R}_{gz}$ value from 20.1 to 389 Å, the accuracy ranged from 1 to 15.3%. Furthermore, from relationships in the literature, $\bar{R}_{gz}$ values were transformed to $\overline{M}_w$. These values of $\overline{M}_w$ were comparable to, but generally less accurate than, $\overline{M}_w$ values from direct, molecular-weight calibration.

INTRODUCTION

In the course of characterizing citrus pectins, it became obvious that existing methods of high-performance size-exclusion chromatography (h.p.s.e.c.) are unsatisfactory, because pectin undergoes concentration-dependent disaggregation[1–3]. Direct molecular-weight characterization of disaggregating macromolecules is often not feasible, because their molecular weight is concentration-dependent, and absolute techniques required for obtaining their molecular weights operate at higher concentration than does h.p.s.e.c. Conventional characterization by universal calibration is often impossible, because intrinsic viscosities cannot be obtained in cases where nonlinear extrapolations to infinite dilution are required. The root-mean-square radius of gyration ($\bar{R}_{gz}$) is a better parameter for calibration, because a linear extrapolation of $\bar{R}_{gz}$ against an unknown concentration is unnecessary.

For a series of pullulans and dextrans having narrow molecular-weight distributions, Kato *et al.*[4] demonstrated the validity of the universal-calibration principle in aqueous h.p.s.e.c Namely, they found that, by plotting log $\bar{R}_{gz}$ against retention time, the data for pullulans and dextrans fall on the same straight line. We now demonstrate the validity of universal calibration with a series of commer-

cially available, well characterized dextran standards having broad molecular-weight distribution and of pullulan standards having narrow molecular-weight distributions. Moreover, molecular weights obtained from empirical relationships between $\bar{R}_{gz}$ and molecular weight are compared with molecular weights from direct calibration

EXPERIMENTAL

Materials — Dextrans were purchased from Pharmacia Chemical Co., Piscataway, NJ, and pullulans, from Polymer Laboratories, Inc., Amherst, MA. Table I contains molecular weight data obtained from these suppliers

Chromatographic analysis. — Chromatography, sample preparation, and determination of column void and total volume have been described elsewhere[5]. H.p.s.e.c. was performed either in a column (30 × 0.39 cm) of Waters E-1000 or E-linear micro-Bondagel. Of a sample (0.3 mg/mL) there was injected 20 μL. The mobile phase, either 0.05M or 0.1M NaCl, was stirred magnetically in the reservoir, and the column was wrapped with soft-foam insulator. The chromatograph was housed in a temperature-controlled room at 23 ±1°. Flow rates were measured by a bubble injected into a calibrated measuring-pipet connected to the exit line of the chromatograph[6]. The pump was set at a nominal flow-rate of 0.5 mL/min. Long-term flow-rates were measured to within ±2% of the nominal value. Over any short period, flow rates were precise to ±0.3%. Generally, 3 consecutive peak-maxima agreed to within 2 s

Peaks emerging from the s.e. chromatograph were detected by differential refractive index (Δr.i.). Analog signals were digitized at the rate of 150 points per min, in a remote location, by a Modcomp 7861 computer equipped with an analog-input subsystem. Chromatograms could be displayed on the cathode-ray tube of an

TABLE I

MOLECULAR WEIGHT STANDARDS

Dextrans					*Pullulans*		
Sample	$\bar{M}_w/\bar{M}_n$[a]	$\bar{M}_w \times 10^{-3}$	$M_p \times 10^{-3}$[b]	WT% $\leq\bar{M}_w$[c]	*Sample*	$\bar{M}_w/\bar{M}_n$	$\bar{M}_w \times 10^{-3}$
T-10	1 63	9 3	7 0	49 0	P-5	1 07	5 8
T-20	1 50	22 3	16 3	70 6	P-10	1 06	12 2
T-40	1 54	44 4	30.0	71 0	P-20	1 07	23 7
T-70	1 65	70 0	40 0	57 0	P-40	1 09	48
T-110	1 39	106	69 0	52 0	P-100	1 10	100
T-250	2 25	253	75 0	69 0	P-200	1 13	186
T-500	2 91	532	140	70 8	P-400	1 12	380
T-2000	—	2000	—	—	P-800	1 14	853

[a] $\bar{M}_w$ is weight average molecular weight, $\bar{M}_n$ is number average molecular weight [b] M_p is molecular weight at peak maximum [c] WT% $\leq \bar{M}_w$ is weight fraction with molecular weight equal to or less than, the weight-average molecular weight

TABLE II

CALIBRATION-CURVE CONSTANTS

Variables				*Constants*[a]					
Col[b]	*Conc.*[c]	*St*[d]	*Sp*[e]	K_1	K_2	b_0	b_1	b_2	b_3
E l.m.b	0 05	P,D	$\bar{R}_{gz}$	0 205	0 728	3 89	−9 055	12 95	−7 35
E-1000	0 05	P,D	$\bar{R}_{gz}$	0 337	0.815	4 04	−8 167	12 02	−6 89
E l m b	0 10	P,D	$\bar{R}_{gz}$	0 258	0 760	4 93	−13 67	20 06	−10 9
E-1000	0.10	P,D	$\bar{R}_{gz}$	0 356	0 811	4 205	−8.332	11.71	−6 54
E l m.b.	0.05	P	$\overline{M}_w$	0 211	0 607	8 568	−18 94	30 92	−19.4
E l m b.	0 05	D	$\overline{M}_w$	0 256	0 675	7 660	−8 411	2 135	3 315
E-1000	0 05	P	$\overline{M}_w$	0 259	0 912	8 075	−12 14	17 89	−10 5
E-1000	0 05	D	$\overline{M}_w$	0 453	0 815	6 749	−3 197	1 382	−1 25
E l m b	0 10	P	$\overline{M}_w$	0 274	0 647	11 42	−33 63	56 60	−34 1
E l m b	0 10	D	$\overline{M}_w$	0 352	0 556	1 156	39 72	−108 3	84 82
E-1000	0 10	P	$\overline{M}_w$	0 356	0 872	8 314	−11 92	15 65	−8 55
E-1000	0 10	D	$\overline{M}_w$	0 497	0 811	6 495	−1 112	−2 037	0 269

[a]Symbols defined by Eqs *1–3* [b]E l m b. = E-linear micro-bondagel column; E-1000 = E-1000 miro-Bondagel column [c]Molar concentration of sodium chloride in mobile phase [d]Standards P = pullulan, d = dextran [e]Size parameters

Admiral Model 5 Dumb Terminal immediately after the run. User-interactive processing of data, including specification of peak base-line, maxima, and integration limits by cursor, was accomplished with software that was developed in-house.

Averages of $\bar{R}_{gz}$ or molecular weight were calculated by transforming partition coefficients (K_{av}), point by point, to either $\bar{R}_{gz}$ or molecular-weight values, and summing the appropriate integrals. Integrations were made by use of a trapezoidal algorithm. Transformations were obtained from the following calibration curves.

$$\ln(Y) = a_0 + a_1 K_{av}, \text{ when } K_1 > K_{av} \quad (1)$$

$$\ln(Y) = b_0 + b_1 K_{av} + b_2 K_{av}^2 + b_3 K_{av}^3, \text{ when } K_1 < K_{av} < K_2 \quad (2)$$

$$\ln(Y) = c_0 + c_1 K_{av}, \text{ when } K_{av} > K_2, \quad (3)$$

where, Y is $\bar{R}_{gz}$ or $\overline{M}_w$.

With the aid of Eqs. *1–3*, the best regression-line is fitted, using values of Y and K_{av}. The constants a_0, a_1, c_1, and c_0 are constrained to make the calibration curve and its first derivative continuous at K_1 and K_2, which are points of intersection between Eqs. *1, 2* and *2, 3*, respectively, were chosen to minimize the sum of the residuals squared. The constants b_0, b_1, b_2, and b_3, governing that portion of the calibration curve with $K_1 < K_{av} < K_2$, were obtained by nonlinear regression, using the Gauss–Newton algorithm. The calibration curves were cubic polynomials with straight lines at the ends. The values of K_1, K_2, a_0, a_1, a_2, and a_3 for the columns used in this study are listed in Table II.

For "narrow" pullulan molecular-weight standards, values of partition

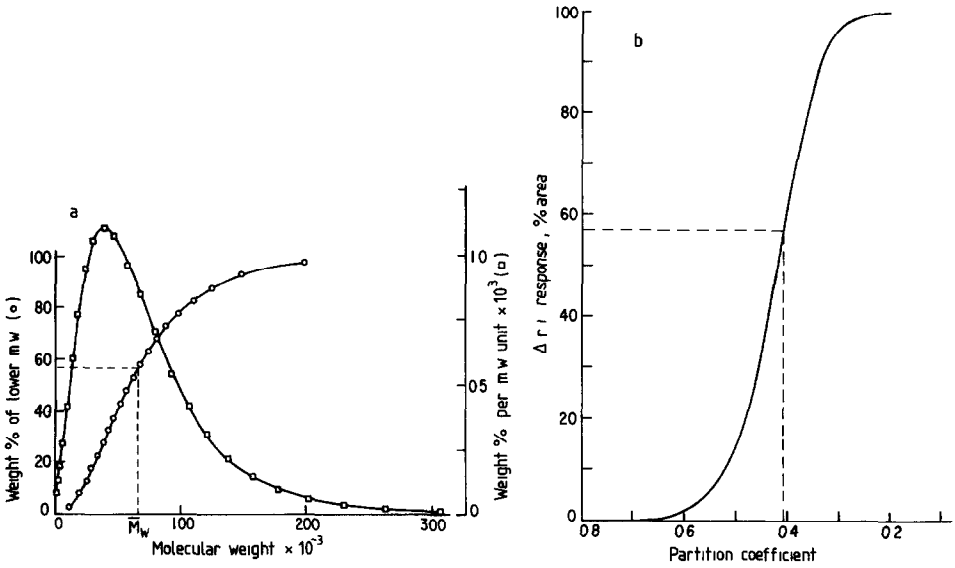

Fig 1 (a) Molecular-weight distribution of dextran T-70 (b) Refractive index integral distribution curve for T-70 dextran from h p s e c on E-linear micro-Bondagel column, with 0 1M NaCl as mobile phase

coefficient corresponding to the peak maximum of the differential, refractive-index trace (Δr.i.) of the chromatogram were correlated with the *z*-average radii of gyration ($\bar{R}_{gz}$) obtained from the literature[4]. Integral distribution curves supplied by the manufacturer gave weight percentage values corresponding to $\overline{M}_w$, the weight-average molecular weight for each broad dextran standard; as shown by Fig. 1a, the value is 57% for T-70 dextran. For each dextran standard, the weight percentages in Table I were equated with area percentages from the Δr.i. trace of the corresponding dextran chromatogram, so that K_{av} on the chromatograms could be correlated with $\overline{M}_w$. A typical, integral-distribution chromatogram for T-70 dextran is shown in Fig. 1b.

THEORY

Eqs. *4–7* are four variations of the universal-calibration principle[9].

$$\log f(V_h) = f(\text{r.t.}) + \text{constant} \quad (4)$$

$$\log [\eta]\overline{M}_w = aV_r + b \quad (5)$$

$$\log [\eta]\overline{M}_n = aV_r + b \quad (6)$$

$$\log \bar{R}_{gz} = aK_{av} + b \quad (7)$$

Eq *4*, the most general statement of the universal calibration principle (u.c.p.) equates some function of the logarithm of the hydrodynamic volume, (V_h), with a function of the column retention-time (r.t.). Eqs. *5* and *6*, the most common state-

ments of the u.c.p., relate the product of the intrinsic viscosity $[\eta]$ and the weight $(\overline{M}_w)$ or number average $(\overline{M}_n)$ molecular weight to the retention volume (V_r). Both of these statements require either viscosities obtained by extrapolation to zero concentration for the unknown, or Mark–Houwink constants[7]. Eq 7, invoking u.c p , enables the $\bar{R}_{gz}$ of the unknown to be obtained from the $\bar{R}_{gz}$ of known standards. If a relationship between $\bar{R}_{gz}$ and molecular weight is available for the unknown, the molecular weight can be obtained. Moreover, in the case of rod-like molecules, the molecular weight of the unknown can be obtained directly from $\bar{R}_{gz}$ measurements, provided that the virtual bond-length of the monomer unit and the monomer-residue weight are known[3,8].

In the case of pullulan, Eq. *8* relates $\overline{M}_w$ to the *z*-average radius of gyration, $\bar{R}_{gz}$ (ref. 4), whereas, in the case of dextran, Eqs. *9* and *10* relate[7] $\overline{M}_w$ to $\bar{R}_{gz}$

Pullulan

$$\overline{M}_w = 37.4\, R_{gz}^{1.68} \tag{8}$$

Dextran

$$R_{gz} \leq 103 \text{ Å}$$
$$\overline{M}_w = 11.9\, R_{gz}^{2} \tag{9}$$

$$R_{gz} > 103 \text{ Å}$$
$$\overline{M}_w = 2.62\, R_{gz}^{2.32} \tag{10}$$

The larger exponents for R_g in Eqs. *9* and *10*, as compared to *8*, indicate that, for isomolecular weights, dextrans are more compact than pullulans.

RESULTS AND DISCUSSION

In Fig. 2a are typical, overlayed chromatograms from separate runs of a series of pullulans chromatographed on a column of micro-Bondagel, E-1000, whereas, in Fig. 2b are comparable data for a series of dextrans. For both sets of chromatograms, the mobile phase was 0.05M NaCl. Comparison of Figs. 2a and 2b confirmed that the dextrans are more polydisperse than the pullulans, and that E-1000 columns discriminate better between the high-molecular-weight standards than the low ones

In Fig. 2c are typical, overlayed chromatograms from separate runs of a series of pullulans chromatographed on a column of E-linear micro-Bondagel, whereas, in Fig. 2d are comparable data for a series of dextrans. For both sets of chromatograms, the mobile phase was 0 1M NaCl. Comparison of these two Figures also confirmed the greater polydispersity of dextrans over pullulans. Furthermore, unlike E-1000 columns, E-linear columns discriminate between the lower-molecular-weight standards better than between the higher ones. Fig. 3a and 3b demonstrate

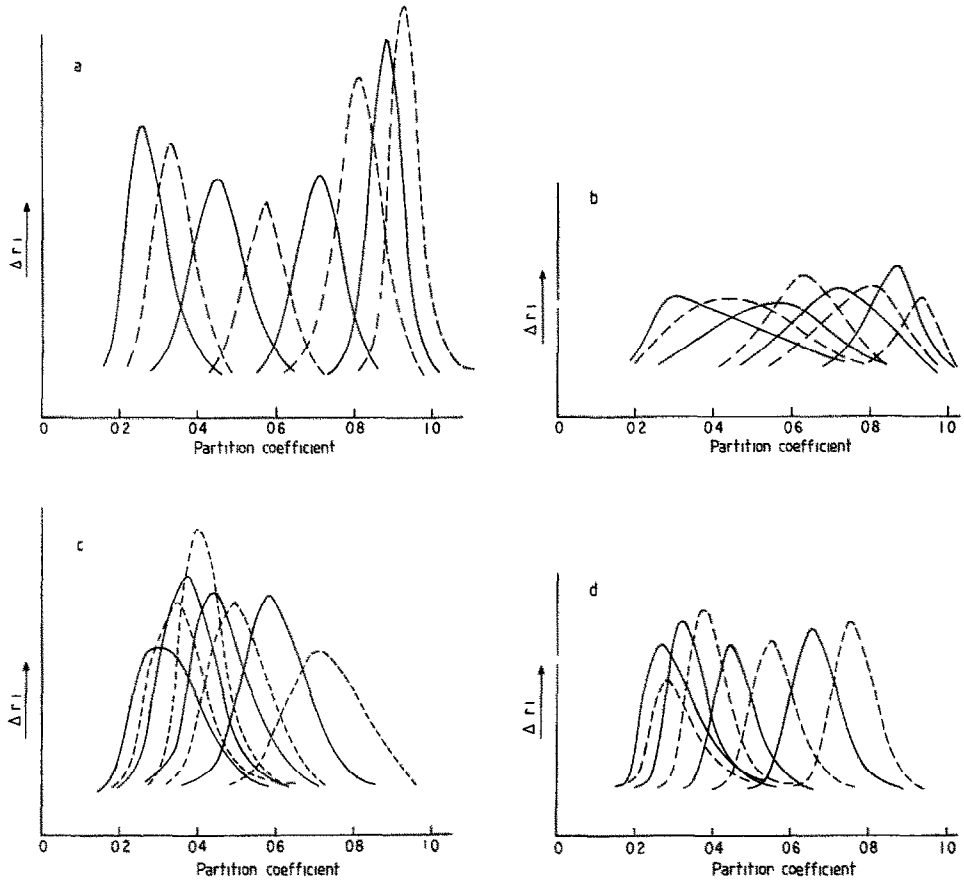

Fig. 2. (a) Overlayed chromatograms of pullulan standards P-800, P-400, P-200, P-100, P-40, P-20, P-10, and P-5. [Dotted and solid lines delineate adjacent chromatograms Column, E-1000 micro-Bondagel; mobile phase, 0.05M NaCl, detector, refractive index] (b) Overlayed chromatograms of dextran standards, T-2000, T-500, T-250, T-100, T-70, T-40, T-20, and T-10. [Dotted and solid lines delineate adjacent chromatograms. Column, E-1000 micro-Bondagel, mobile phase. 0.05M NaCl; detector, refractive index.] (c) Overlayed chromatograms of pullulan standards P-800, P-400, P-200, P-100, P-40, P-20, P-10, and P-5. [Dotted and solid lines delineate adjacent chromatograms. Column, E-1000 micro-Bondagel, mobile phase, 0.1M NaCl; detector, refractive index.] (d) Overlayed chromatograms of dextran standards T-2000, T-500, T-250, T-110, T-70, T-40, T-20, and T-10 [Dotted and solid lines delineate adjacent chromatograms. Column, linear micro-Bondagel; mobile phase, 0.1M NaCl; detector, refractive index]

that, for either the E-1000 or E-linear column, in 0.05 or 0.1M NaCl, a combination of narrow pullulan standards and broad dextran standards gives universal calibration when $\bar{R}_{gz}$ values are assigned as described in the Experimental section. By way of comparison, as shown by Figs. 4a and 4b, log $\overline{M}_w$ against K_{av} plots, on both columns, and in both mobile phases, gave separate calibration curves for each polysaccharide. In the case of the pullulans, it was assumed that the K_{av} at peak maximum correlated with $\overline{M}_w$, whereas, in the case of the dextrans, K_{av} at $\overline{M}_w$ was obtained by matching areas as described in the Experimental section.

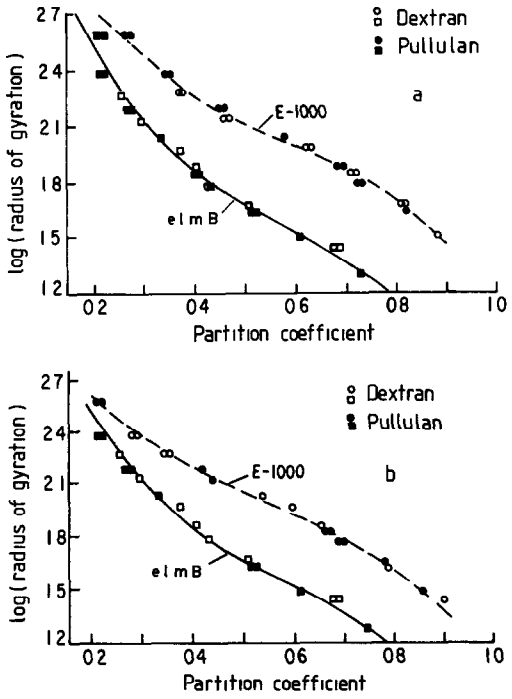

Fig. 3 (a) Universal calibration on columns of E-linear and E-1000 micro-Bondagel in 0 05M NaCl. [All standards in Table I, except T-2000 dextran.] (b) Universal calibration on columns of E-linear and E-1000 micro-Bondagel in 0 1M NaCl [All standards in Table I, except T-2000]

To check on the validity of the universal calibration curves of Figs. 3a and 3b, $\bar{R}_{gz}$ was calculated from the chromatograms of each standard according to Eq. *11*.

$$\bar{R}_{gz} = \sum_i C_i R_{gi}^2 / \Sigma_i C_i R_{gi} \qquad (11)$$

where C_i is the concentration of the ith species.

For the pullulans, as indicated by the data of Table III, the column of E-linear micro-Bondagel (e.l.m.b.) gave better agreement with the literature values for the samples near the total volume, V_t, whereas the E-1000 columns appeared to give better agreement with literature values for samples near the void value V_0. Salt concentration in the mobile phase appeared to have no effect on $\bar{R}_{gz}$; thus, values for the two salt concentrations (*i.e.*, 0.05 or 0.1M NaCl) were combined. In the case of the dextrans, comparison of literature values with measured values gave no appreciable differences with column pore-size distribution or salt concentration in the mobile phase. Therefore, for each dextran sample in Table III, the twelve values of $\bar{R}_{gz}$ were averaged. For polysaccharides that are eluted near V_t, e.l m.b. columns appear to discriminate better between peaks than E-1000 columns (see Figs. 2a–d), whereas, for those polysaccharides that are eluted near V_0, E-1000

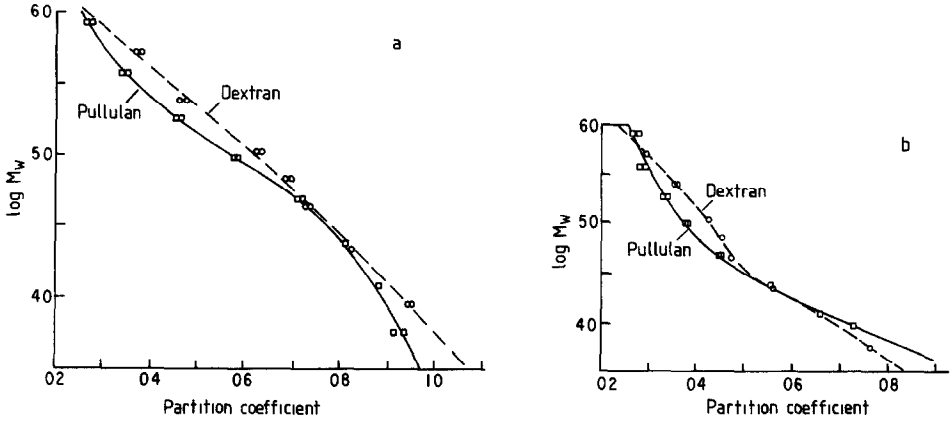

Fig 4 (a) Weight-average molecular weight calibration on column of E-1000 micro-Bondagel in 0 05M NaCl [All standards in Table I, except T-2000 dextran.] (b) Weight-average molecular weight calibration on column of E-1000 micro-Bondagel in 0 1M NaCl. [All standards in Table I, except T-2000 dextran]

columns appear to discriminate better between peaks than do e.l.m.b., columns. The $\overline{R}_{gz}$ values of the pullulans appear to be more affected by poor resolution at both ends of the size range than are those of the dextrans.

The values of $\overline{R}_{gz}$, for pullulan, in Table III, were transformed into $\overline{M}_w$ values by utilizing Eq. *8*, whereas R_{gz} values for dextrans in Table III were transformed into $\overline{M}_w$ values by utilizing Eqs. *9* and *10*. Pullulan and dextran $\overline{M}_w$ values so calculated are given in Table IV. Values of $\overline{M}_w$ from direct molecular-weight calibration (*e.g.*, from calibration curves in Figs. 4a and 4b) were compared with $\overline{M}_w$ values from R_{gz}, even though direct calibration curves contain half of the calibration standards contained in the universal calibration curves These comparisons revealed that $\overline{M}_w$ from direct calibration ($\overline{M}_w$ cal.) were closer to values provided by the supplier than were values from universal calibration (univ. cal.). Experimental errors in Eq. *8–10* probably account for the additional error in $\overline{M}_w$ from universal calibration, as compared to $\overline{M}_w$ from direct calibration. The $\overline{M}_w$ of T2000 in Table I is only an approximate value. Our data indicate that, for T2000, $\overline{M}_w$ is considerably lower than the nominal value of 2×10^6.

For the pullulans, it was assumed that the molecular weight at peak maximum did not differ appreciably from $\overline{M}_w$. The validity of this assumption was checked by comparing molecular weights at peak maximum with those from the definition of $\overline{M}_w$. The data in Table IV confirmed that the assumption is a good one within experimental error (*cf.*, columns 11 and 15).

For the dextrans, $\overline{M}_w$ was correlated with the appropriate K_{av} value by matching areas under the integral weight-distribution curve (*e.g.*, Fig. 1a) with the same integral area obtained from the $\Delta r.i.$ trace the chromatogram (*e.g.*, Fig. 1b). This method of matching areas was checked by calculating the number-average molecular weight obtained from calibration curves such as are found in Figs. 4a and 4b.

TABLE III

RADII OF GYRATION (Å)

Pullulans						*Dextrans*			
Sample	*Lit value*[a]	*E.l.m b.*[b]	*Rel s.d.*[c]	*E-1000*	*Rel. s d*[c]	*Sample*	*Lit value*[d]	*E-1000-e l m b*[e]	*Rel s d*[f]
P-5	20 1	21 3	0 5	26 3	2 3	T-10	27 6	28 1	2 0
P-10	31 2	31.8	0.5	33.9	2 5	T-20	42.8	42.0	1.9
P-20	46.3	44.9	0 5	46 7	11.3	T-40	60.5	62 8	1 8
P-50	70.4	70 0	0.4	74 1	1 0	T-70	75.7	82.7	3 7
P-100	109	115	0.9	109	0.4	T-110	95 6	97 7	2 0
P-200	156	180	4.5	167	1 9	T-250	139	155	3 6
P-400	241	271	4.0	168	2 1	T-500	191	205	2.5
P-800	389	414	1.9	375	1.7	T-2000	338	268	6 3

[a]Calculated from Eq *8*. [b]Measured on E-linear micro-Bondagel column (e l m.b.) [c]Percentage relative standard deviation of 6 determinations (1 column × 2 mobile phase × 3 replicates) [d]Calculated from Eq. *9* and *10* [e]Value from e l m b and E-1000 columns combined [f]Percentage relative standard deviation of 12 determinations (2 column × 2 mobile phase concentrations × 3 replicates)

TABLE IV

EXPERIMENTAL MOLECULAR WEIGHTS ($\times 10^{-3}$)

Dextrans									*Pullulans*						
Sample	$\overline{M}_w$ *Cal*[a]	*R s d*[b]	*U c*[e]	*R s d*[b]	$\overline{M}_n$[c]	*R s d*	M_p[d]	*R s d*	*Sample*	$\overline{M}_w$ *cal*[a]	*R s d*[b]	*U c*[e]	*R s d*[b]	M_p[d]	*R s d*[b]
T-10	10 1	3 0	9 4	4 0	8 6	5 8	9 7	4 1	P-5	5 7	3 7	5 4	12	5 7	9 1
T-20	19 9	3 0	21 0	3 8	15 8	1 9	15 8	30 0	P-10	11 5	3 3	13 7	2 9	12 6	2 7
T-40	42 7	4 7	46 9	5 3	26 9	2 6	31 6	8 2	P-20	23 3	12 0	23 3	14	23.5	12 4
T-70	76 3	3 4	81 8	3 7	42 5	3 1	64 2	9 2	P-50	48 9	1 3	49 9	1 4	48 2	3 2
T-110	111	3 6	114	4 0	67 6	4 1	101	9 9	P-100	99 2	1 2	105	0 9	95 8	3 1
T-250	243	3 7	330	8 9	107	18	161	12.4	P-200	215	4 7	220	5 0	195	6 1
T-500	391	2 6	638	6 0	174	5 2	297	12 8	P-400	470	6 1	459	5 5	465	10 4
T-2000	690	5 9	1221	19 3	215	13	754	13 3	P-800	780	3 3	847	3 0	807	10 0

[a]Obtained with log $\overline{M}_w$ calibration [b]Percentage relative-standard deviation of 12 determinations (2 columns × 2 mobile phase × 3 replicates), except for P-5 + P-10 standards, which have values for e l m b column only (1 column × 2 mobile phases × 3 replicates) or 6 determinations [c]Number-average molecular weight obtained with log $\overline{M}_w$ calibration [d]Molecular weight at peak maximum [e]Universal calibration obtained with log R_{gz} calibration, employing all standards but T-2000

Generally, these values of $\overline{M}_n$ agree more closely with supplier values than do experimental values of $\overline{M}_w$ compared with supplier values of $\overline{M}_w$. Surprisingly, comparison of the molecular weight at peak maximum (M_p) values supplied by the manufacturer (see Table I) were very different from the values of M_p found by h.p.s.e.c. (see Table IV).

The only exceptions were the T-20 and T-40 dextrans, for which there was good agreement between the two values. Generally, our M_p values from h.p.s.e.c. were higher those provided by the supplier. We assume that the polydispersity of the pullulan samples was sufficiently small that $\overline{M}_w$ values supplied by the manufacturer could be associated with the peak maximum observed by h.p.s.e.c. This assumption was proved valid by the data given in Table IV. Values of $\overline{M}_w$ from direct molecular-weight calibration agree within experimental error with values obtained from the peak maximum.

CONCLUSION

In conclusion, we have shown that $\overline{M}_w$ correlates well with $\bar{R}_{gz}$ for universal calibration by combining broad and narrow standards in the same calibration curve. Furthermore, well characterized, commercial samples are available for universal calibration of polysaccharides. In addition, direct molecular-weight calibration is somewhat more accurate than are molecular weights from universal calibration. Finally, $\bar{R}_g$ is measured more accurately than $\overline{M}_w$ by universal calibration

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