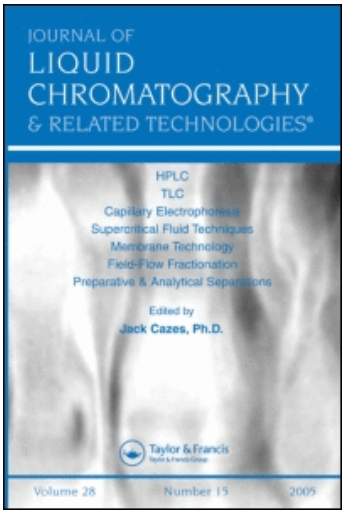


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# Characterization of Dextrans by Size Exclusion Chromatography Using DRI/LALLSP Detector System

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# CHARACTERIZATION OF DEXTRANS BY SIZE EXCLUSION CHROMATOGRAPHY USING DRI/LALLSP DETECTOR SYSTEM

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## ABSTRACT

Herein is reported an experimental investigation of the molecular weight characterization of dextrans by aqueous SEC with a DRI/LALLSP detector system. Methodology for the determination of the molecular weight calibration curve and peak broadening parameter $\sigma^2$ (variance of a Gaussian instrumental spreading function) across the chromatogram have been developed. A blend of dextran standards permits, with one injection, measurement of the molecular weight calibration curve over a wide range of retention volumes. Measurements with salt-free water as mobile phase have confirmed that some dextran chains may have a negative charge.

## INTRODUCTION

The use of an LALLSP detector with SEC is a relatively recent development[1-8]. In fact, to date, use of aqueous SEC with a LALLSP detector with dextrans has been reported only once[8]. Dextran was chosen as a test solute to develop the technique as previous studies showed that aqueous SEC of these polymers was

relatively straightforward with the absence of significant ion exclusion and adsorption and with a wide range of well characterized broad MWD standards available commercially[9]. Generalized equations which correct for dispersion in the detector cell and which are applicable for the case of a nonlinear molecular weight calibration curve and peak broadening parameters which change with retention volume and molecular size have recently been published[10,11]. These correction equations form the basis for the method of determining the molecular weight calibration curve and peak broadening parameters across the chromatogram of a broad MWD polymer developed herein.

## THEORY

It is assumed that Tung's integral equation adequately describes peak broadening in the aqueous SEC of dextrans.

$$F(v) = \int_0^\infty w(y)\, G(v,y)\, dy \tag{1}$$

A distribution function w(v,y) which describes peak broadening in the detector cell is now defined. w(v,y) dvdy is the area under the detector response at retention volume, v-v+dv due to species with mean retention volume, y-y+dy. This distribution function has the following properties.

$$w(v,y) = w(y)\, G(v,y) \tag{2}$$

$$\int_0^\infty w(v,y)dy = F(v) \tag{3}$$

$$\int_0^\infty w(v,y)dv = w(y) \tag{4}$$

It is now assumed that the instrumental spreading function, G(v,y) is Gaussian with a variance, $\sigma^2(v)$ which depends on retention volume. It is further assumed that w(v,y) is Gaussian and of the form.

$$w(v,y) = \frac{F(v)}{\sqrt{2\pi\ \bar{\sigma}(v)^2}} \exp\left((v - \bar{y}(v))^2/2\ \bar{\sigma}(v)^2\right) \quad (5)$$

The mean $\bar{y}(v)$ and variance $\bar{\sigma}(v)^2$ of $w(v,y)$ can be related to $\sigma(v)^2$ the variance of the instrumental spreading function and to $D_2(v)$ the local slope of the molecular weight calibration curve which is given by

$$M(y) = D_1(v) \exp(- D_2(v)y) \quad (6)$$

as follows

$$\bar{y}(v) = v + \frac{1}{D_2(v)} \ln\left(\frac{F(v + D_2(v)\ \sigma(v)^2)}{\sqrt{F(v-D_2(v)\sigma(v)^2)\cdot F(v+D_2(v)\sigma(v)^2)}}\right) \quad (7)$$

$$\bar{\sigma}(v)^2 = \sigma(v)^2 + \frac{1}{D_2^2(v)} \ln\left(\frac{F(v-D_2(v)\sigma(v)^2)\cdot F(v+D_2(v)\sigma(v)^2)}{F(v)^2}\right) \quad (8)$$

It is clear that $\bar{y}(v)$ and $\bar{\sigma}(v)^2$ should not depend on $D_2(v)$ and indeed this has been shown[11]. For computational convenience equations (7) and (8) are used with any convenient value of $D_2(v)$. Once $\bar{y}(v)$ and $\bar{\sigma}(v)^2$ are known, the corrected response for the mass concentration detector is given by

$$w(v) = F(v)(\sigma(v)/\bar{\sigma}(v))\exp(-(v - y(v))^2/2\bar{\sigma}(v)^2) \quad (2a)$$

The correction equation for the weight-average molecular weight in the detector cell follows.

$$\frac{\bar{M}_w(v,uc)}{M(v)} = \frac{F(v - D_2(v)\sigma(v)^2)}{F(v)} \exp((D_2(v)\sigma(v))^2/2) \quad (9)$$

This equation is later used to determine $\sigma(v)$ across the chromatogram and then equations (7), (8) and (9) are used to determine the corrected detector response, $w(v)$.

## EXPERIMENTAL

Operational details of the aqueous SEC chromatograph employed follow:

Packing: 3/8 inch ID columns (4 foot long approx.) dry-packed with CPG10 200/400 and 120/200 mesh glass packings

Mobile phase: 0.05 M $K_2HPO_4$ (NaOH to pH = 7.0) in deionized distilled water

Flowrate: 1ml/min.

Sample size: 0.4 ml at 0.5-1.0 wt% solution

Temperature: ambient

Inline filter: 0.22 micron Millipore

Detectors: Waters R-403 DRI and Chromatix KMX-6 with angle 6-7° and field stop 0.15.

Polymer Samples: Pharmacia dextran standards and special blends of these standards with a desired molecular weight distribution.

A published specific refractive index increment (dn/dc = 0.1378) for dextran in 0.05 M $K_2HPO_4$ (pH = 7.0) at a wavelength $\lambda$ = 632.8 nm was used in this investigation[12]. A second virial coefficient ($A_2$ = 0.41 x $10^{-3}$ ml/gmole) was determined for dextran standard T250. Setting $A_2$ = 0 in the measurement of $\bar{M}_w(v,uc)$ introduced an error of less than 2%, however. This correction for polymer solute concentration was therefore neglected for all the dextran samples characterized.

## RESULTS AND DISCUSSION

To optimize pore size for the dextran standards, single columns containing one pore size were calibrated. Molecular weight calibration curves for these single columns of different length are shown in Fig. 1. In agreement with a previous

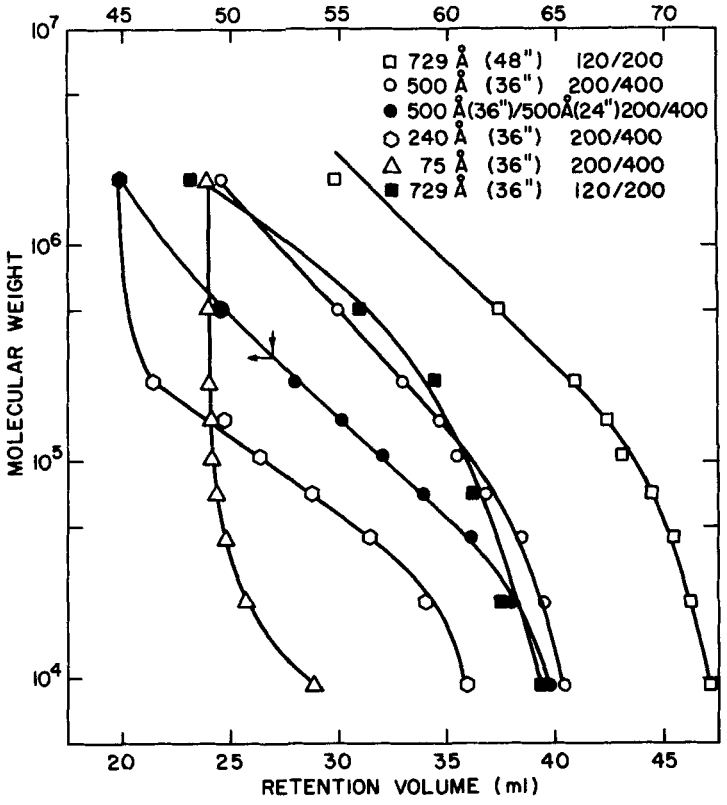


Figure 1. Molecular weight calibration curves obtained using Pharmacia dextran standards with CPG10 packings, mobile phase - 0.05M $K_2HPO_4$(pH=7.0) at 1.0 ml/min.

investigation[13] pore sizes of 700Å, 500Å, 240Å and 75Å gave suitable peak separation and were used in this investigation. With a proper choice of column length for each pore size a column combination was selected which gave an almost linear molecular weight calibration curve over a wide molecular weight range. To construct the calibration curve the root-mean-square ($\sqrt{\bar{M}_N \cdot \bar{M}_w}$) or $\bar{M}_{rms}$ molecular weight is assigned to the peak position. This procedure is in general not valid for all broad MWD standards, however it does provide a good first guess of the molecular weight

calibration curve. The molecular weight calibration curves for the chosen column combination using $\bar{M}_{rms}$ at the peak position and SEC/DRI/LALLSP are shown in Fig. 2. The $\bar{M}_{rms}$ was calculated using $\bar{M}_N$ and $\bar{M}_w$ values supplied by Pharmacia. The SEC/DRI/LALLSP calibration curve was found by setting $M(v) = \bar{M}_w(v,uc)$ at the peak position of the broad MWD standards. An examination of equation

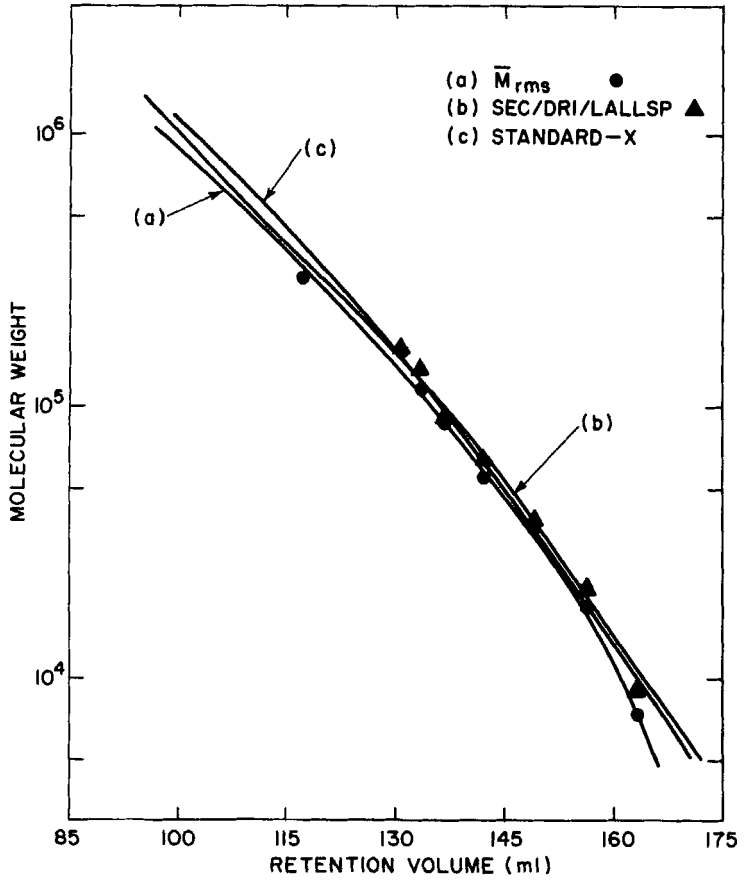


Figure 2. Molecular weight calibration curves for optimized column combination of CPG10 packings using Pharmacia dextran standards ($\bar{M}_{rms}$, SEC/DRI/LALLSP) and dextran standard-X (SEC/DRI/LALLSP).

(9) reveals that the correction for peak broadening is small near the peak position justifying the assumption, $M(v) = \bar{M}_w(v,uc)$.

Typical responses obtained from SEC/DRI/LALLSP are shown in Fig. 3. The noise in the LALLSP response clearly shows that particulate matter and deformable micelles pass through the inline 0.22 μm filter and enter the detector cell. To obtain a relatively smooth LALLSP response the mobile phase is pumped continuously at a low flowrate (0.1 ml/min) when the chromatograph

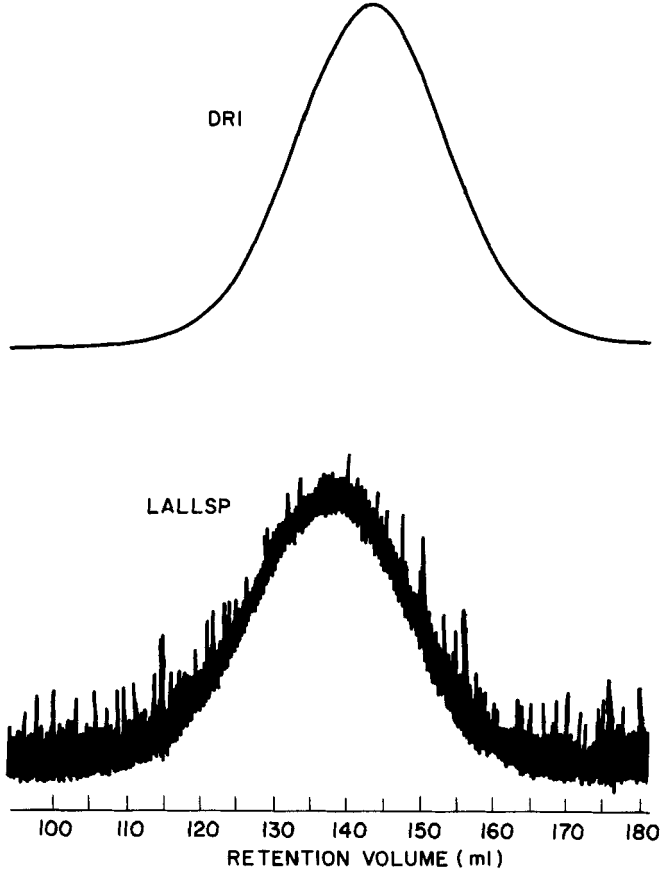


Figure 3. SEC/DRI/LALLSP chromatograms for Pharmacia dextran standard T70.

is not being used. To prepare the system for use the flowrate is increased to the desired level (1 ml/min in this study) and run for 5-6 hours before a polymer sample is injected. We have observed that with the initial startup of an SEC/DRI/LALLSP from zero flowrate, it can take up to a week of operation before an LALLSP response has an acceptable signal-to-noise ratio.

The calculational procedure for finding $\sigma(v)$ across the chromatogram of a single broad MWD standard is now discussed. The calibration curve $M(v)$ and its slope $D_2(v)$ have already been determined (SEC/DRI/LALLSP in Fig. 2). $\bar{M}_w(v,uc)$ is measured by SEC/DRI/LALLSP leaving one unknown $\sigma(v)$. $\sigma(v)$ was found using a single-variable search (Fibonnaci search in this study) across the chromatogram. Typical results for the two detector responses and $\sigma(v)$ are shown in Fig. 4. It is encouraging that the magnitude of $\sigma(v)$ per unit column length and the dependence of $\sigma(v)$ on



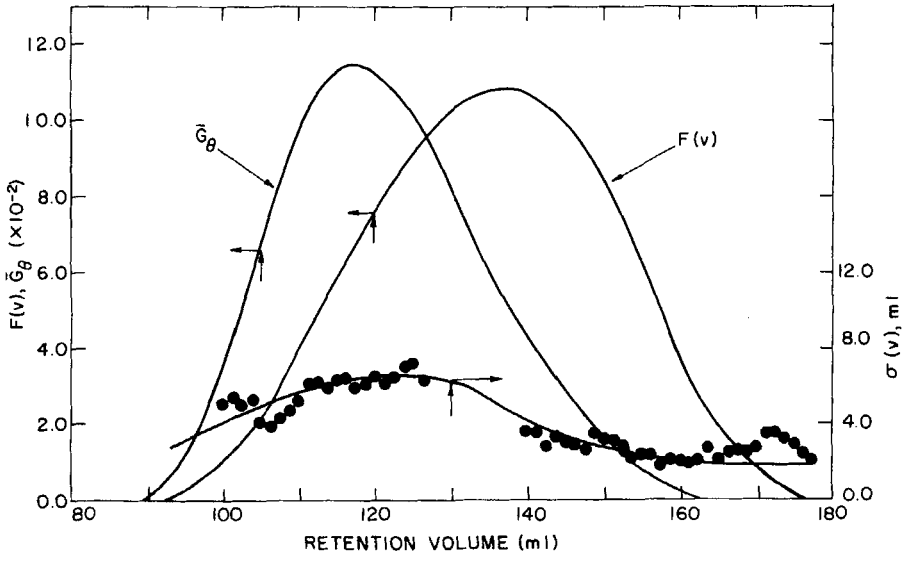


Figure 4. SEC/DRI/LALLSP chromatograms for a blend of Pharmacia dextran standards (71% T250 and 29% T40) and calculated peak broadening parameter $\sigma(v)$.

retention volume is in general agreement with that observed by Basedow et al.[13]

We now discuss a technique of finding the true molecular weight calibration curve using one very broad MWD standard having a designed chromatogram shape. The broad MWD standard is obtained by mixing a number of broad MWD Pharmacia standards to obtain the desired chromatogram shape. An observation of equation (9) reveals the required shape. One would like the ratio $\bar{M}_w(v,uc)/M(v)$ to be as close to unity as possible over a wide range of retention volumes so that an $\bar{M}_w(v,uc)$ measurement gives a point on the molecular weight calibration curve. The exponential factor is always greater than unity and it is therefore desirable that the pre-exponential factor be less than unity hopefully to almost compensate and give a ratio close to one. A very broad MWD standard (STANDARD-X) was made by mixing T250(53wt.%), T40(29wt.%) and T10(18wt.%). The SEC/DRI/LALLSP responses are shown in Fig. 5. Standard X is now used to determine the molecular weight calibration curve. The molecular weight calibration curve is taken to be linear over a narrow retention volume range ($v_i$ to $v_{i+1}$). Equation (9) is now rearranged to give

$$\ln\left(\frac{\bar{M}_w(v_i,uc)}{\bar{M}_w(v_{i+1},uc)}\right) = D_2(v_{i+1} - v_i) + \frac{D_2^2}{2}(\sigma_i^2 - \sigma_{i+1}^2) + \ln\left\{\frac{F(v_i - D_2\sigma_i^2)\,F(v_{i+1})}{F(v_{i+1} - D_2\sigma_{i+1}^2)F(v_i)}\right\} \tag{9a}$$

Given $\sigma_i$ across the chromatogram, one can solve equation (9a) for $D_2$ and then equation (9) for $D_1$ across the chromatogram. The molecular weight calibration curve is thus established. The drawback of having to know $\sigma_i$ is not a serious one. In fact, the determination of the molecular weight calibration curve with specially prepared Standard-X is very insensitive to $\sigma_i$ as is shown in Table 1. The first column of $\bar{M}_w(v,uc)/M(v)$ values were

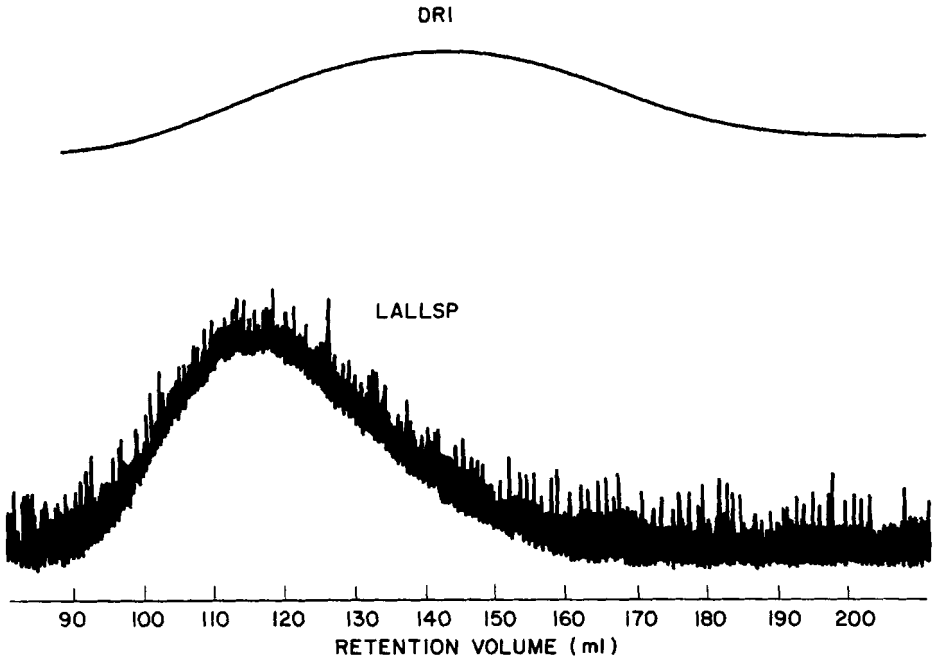


Figure 5. SEC/DRI/LALLSP chromatograms for a blend of Pharmacia dextran standards called standard-X (53% T250, 29% T40 and 18% T10).



obtained using the previously measured $\sigma(v)$ values. To illustrate the lack of sensitivity of this ratio to $\sigma(v)$, values of up to 50% lower and 50% higher were employed and $\bar{M}_w(v,uc)/M(v)$ calculated. These calculations clearly indicate that the designed shape of the chromatogram for Standard-X has served the purpose of giving $\bar{M}_w(v,uc)/M(v)$ ratios close to unity over a wide range of retention volumes. They also indicate that when Standard-X is used to obtain the molecular weight calibration curve a precise knowledge of $\sigma(v)$ is not required. In other words peak broadening parameters are not required when using Standard-X to obtain the molecular weight calibration curve ($\sigma(v)$ can be set equal to zero with small error).

TABLE 1

Standard-X - Sensitivity to Peak Broadening Parameter

| Retention volume (ml) | $\bar{M}_w(v,uc)/M(v)$ | | | | |
|---|---|---|---|---|---|
| | $\sigma(v)$ actual | 0.8 $\sigma(v)$ | 0.5 $\sigma(v)$ | 1.2 $\sigma(v)$* | 1.5 $\sigma(v)$* |
| 110.6 | 0.922 | 0.951 | 0.981 | - | - |
| 115.6 | 0.915 | 0.952 | 0.983 | - | - |
| 120.6 | 0.972 | 0.983 | 0.994 | 0.961 | - |
| 125.6 | 1.011 | 1.006 | 1.002 | 1.019 | 1.036 |
| 130.6 | 1.036 | 1.024 | 1.010 | 1.052 | 1.076 |
| 135.6 | 1.028 | 1.018 | 1.007 | 1.032 | 1.057 |
| 140.6 | 1.032 | 1.021 | 1.008 | 1.045 | 1.067 |
| 145.6 | 1.032 | 1.020 | 1.008 | 1.045 | 1.072 |
| 150.6 | 1.026 | 1.016 | 1.006 | 1.038 | 1.060 |
| 155.6 | 1.018 | 1.011 | 1.004 | 1.026 | 1.041 |
| 160.6 | 1.017 | 1.011 | 1.004 | 1.024 | 1.038 |
| 165.6 | 1.018 | 1.012 | 1.005 | 1.026 | 1.040 |
| 170.6 | 1.019 | 1.012 | 1.005 | 1.027 | 1.042 |
| 175.6 | 1.025 | 1.016 | 1.006 | 1.036 | 1.059 |
| 180.6 | 1.015 | 1.010 | 1.004 | 1.021 | 1.031 |

* Calculations at low retention volumes were not possible because of excessively large $\sigma(v)$.

The molecular weight calibration curve obtained using Standard-X was fitted by equation (10)

$$\ln M(v) = 17.80 - 1.695\times10^{-2}\ V - 2.158\times10^{-4}\ V^2 \qquad (10)$$

and is shown plotted in Fig. 2.

The use of the analytical solution for w(v) (given as equation (2a)) for Pharmacia standard T70 is shown in Fig. 6. As

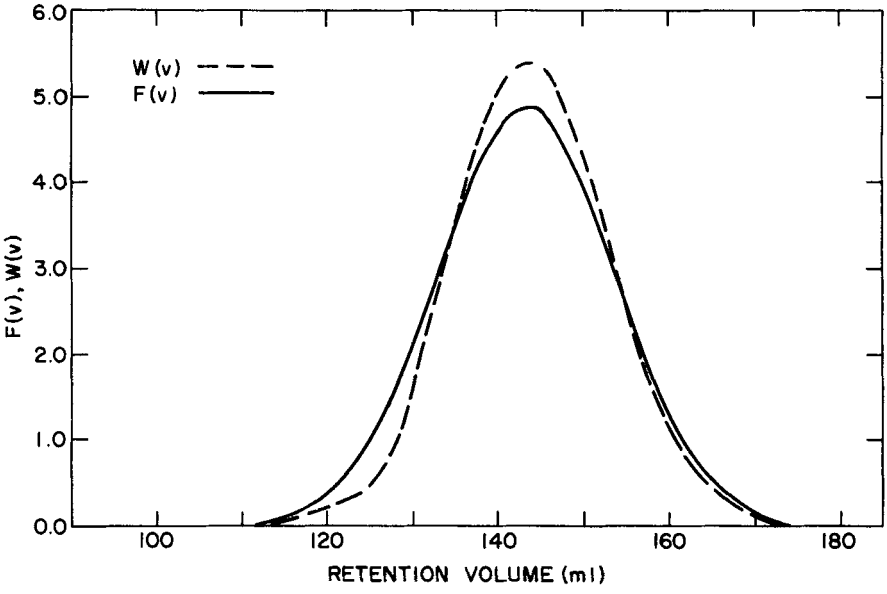


Figure 6. SEC/DRI chromatograms (raw and corrected for peak broadening) for Pharmacia dextran standard T70.



expected a greater correction for peak broadening is required at the high molecular weight end of the chromatogram. The $\sigma(v)$ values used for the correction are those shown in Fig. 4.

Molecular weight characterization data ($\bar{M}_N$ and $\bar{M}_w$) measured for a number of Pharmacia dextran standards are presented in Table 2. Molecular weight averages for the whole polymers corrected for peak broadening ($\bar{M}_N(c)$, $\bar{M}_w(c)$) were calculated using two calculation paths.

The first calculation path employed equations (11a) and (11b)

$$\bar{M}_N(c) = \left(\int_0^\infty F_N(v)/\bar{M}_N(v,uc)dv\right)^{-1} \tag{11a}$$

$$\bar{M}_w(c) = \int_0^\infty F_N(v)\bar{M}_w(v,uc)dv \tag{11b}$$

TABLE 2

Molecular Weight Characterization of Dextrans by SEC/DRI/LALLSP Using Two Calculation Paths (equations (11a,b) and equations (13a,b)).

| Sample | $\bar{M}_N$ | $\bar{M}_w$ | $\bar{M}_N$ | $\bar{M}_w$ | $\bar{M}_N$ | $\bar{M}_w$ |
|---|---|---|---|---|---|---|
| | Pharmacia | | (Equations (11a,b)) | | (Equations (13a,b)) | |
| | | $\times 10^{-3}$ | | $\times 10^{-3}$ | | $\times 10^{-3}$ |
| T250 | 112.5 | 231.0 | 100.3 | 226.0 | 90.0 | 256.0 |
| T150 | 86.0 | 154.0 | 76.9 | 141.0 | 69.6 | 139.1 |
| T110 | 76.0 | 106.0 | 79.2 | 100.5 | 72.3 | 100.3 |
| T70 | 42.5 | 70.0 | 43.0 | 70.4 | 40.1 | 69.4 |
| T40 | 28.9 | 44.4 | 25.6 | 42.7 | 25.7 | 42.2 |
| T20 | 15.0 | 22.3 | 16.9 | 22.7 | 14.7 | 22.7 |



where $F_N(v)$ is the normalized DRI detector response and $\bar{M}_w(v,uc)$ is the weight average molecular weight measured by SEC/DRI/LALLSP with no corrections for peak broadening. $\bar{M}_N(v,uc)$ was calculated using equation (12).

$$\bar{M}_N(v,uc) = \bar{M}_w(v,uc)\left(\frac{F(v)^2}{F(v+D_2(v)\sigma(v)^2)\cdot F(v-D_2(v)\sigma(v)^2)}\right)$$

$$\exp\left(-(D_2(v)\sigma(v))^2\right) \qquad (12)$$

where $D_2(v)$ is obtained from the molecular weight calibration curve and $\sigma(v)^2$ with the peak broadening calibration procedure described earlier.

The second calculation path employed equations (13a) and (13b).

$$\bar{M}_N(c) = \left(\int_0^\infty w(v)M(v)^{-1}dv\right)^{-1} \qquad (13a)$$

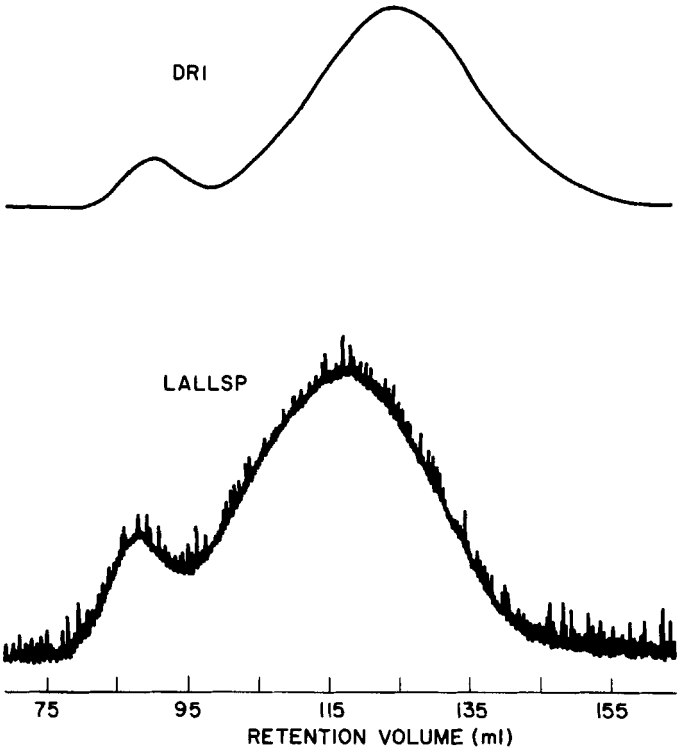


Figure 7. SEC/DRI/LALLSP chromatograms for Pharmacia dextran standard T150 with water free of salt as the mobile phase showing the so called "ghost" peak.



$$\bar{M}_w(c) = \int_0^\infty w(v)M(v)dv \qquad (13b)$$

where w(v) is the DRI detector response corrected for peak broadening via equation (2a) and normalized. The values for $\bar{M}_N$ and $\bar{M}_w$ found using the two calculation paths are in good agreement (probably within experimental error). The recommended procedure is to use SEC/DRI/LALLSP to determine the molecular weight calibration curve and the peak broadening parameter $\sigma(v)^2$ across the retention volume range of interest as described and then for

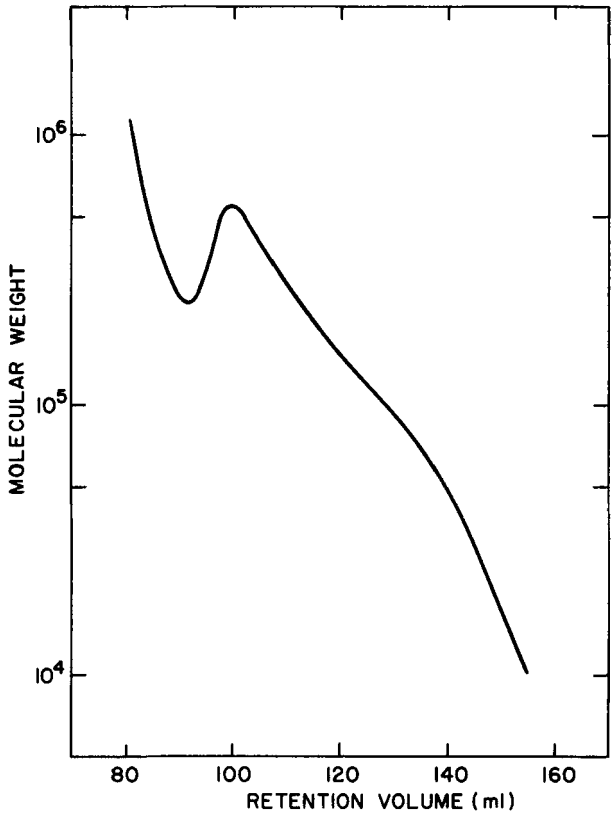


Figure 8. $\bar{M}_w(v,uc)$ measured by SEC/DRI/LALLSP for Pharmacia dextran standard T150 with water free of salt as the mobile phase showing the effect of the "ghost" peak.



further dextran characterizations to use SEC/DRI and equations (13a,b). The use of LALLSP online with SEC need only be made for the initial calibration.

Finally, it was decided to investigate the so called "ghost" peaks found with dextrans and SEC when pure water free of salt is used as mobile phase[9]. Typical detector responses for SEC/DRI/LALLSP using water free of salt as mobile phase are shown in Fig. 7. The apparent high molecular weight peak had $\bar{M}_w(v,uc)$ values which were smaller than expected on the basis of a molecular size separation (see Fig. 8). Apparently, some dextran

chains may have a negative charge and experience ion exclusion from the CPG10 packing.

## SUMMARY

A methodology for the use of aqueous SEC/DRI/LALLSP with dextrans has been developed. This includes procedures for the determination of the molecular weight calibration curve using a broad MWD standard with a designed chromatogram shape. Also included are procedures for the determination of the peak broadening parameter, $\sigma^2$ (variance of a Gaussian instrumental spreading function) over a wide retention volume range using a single broad MWD standard.



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