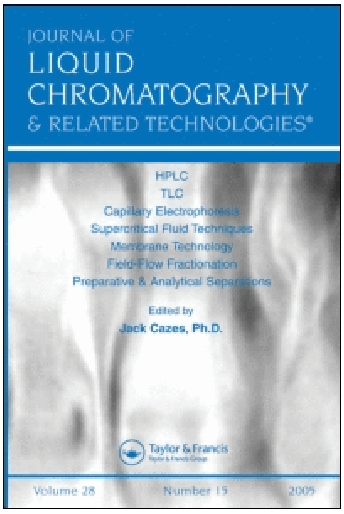

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SIZE EXCLUSION CHROMATOGRAPHY CALIBRATION ASSESSMENT UTILIZING COUPLED MOLECULAR WEIGHT DETECTORS

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ABSTRACT

This paper shows that the usual method of representing an SEC calibration curve by a single polynomial curve may often be inadequate with new high resolution columns. Data points wind about the fitted line. The significant magnitude and systematic nature of these deviations clearly appear when a plot of residuals is derived from the conventional calibration curve and expressed in terms of the percent error in molecular weight. The deviation of the calibration data from the fitted line was approximately 10% for the conventional molecular weight and intrinsic viscosity calibration curves. It became 20% for the universal calibration curve. LALLS and DV detectors were used together with the DRI detector to provide evidence that the calibration curve deviations were due to the column packings and not due to some other cause (e.g., vendor values of molecular weight). Use of a polynomial fit to a portion of the curve corresponding to the retention volume range of an unknown was used to show the significant improvement in results which occurred when the calibration variations were taken into account. At present, use of many individual narrow standards is necessary to elucidate the effect.

INTRODUCTION

New column technology in size exclusion chromatography (SEC) is increasingly directed at maintaining high resolution while providing a linear calibration curve. One of the primary ways of accomplishing this aim is tailoring mixtures of different pore size gels. The objective of this paper is to show that this approach sometimes results in undesirable variations in the fractionation characteristics of the columns. A method of detecting these variations is shown and ways of accommodating them are examined.

THEORY

Plots of Residuals for Examining Fractionation

The conventional calibration curve in SEC is determined by injecting a series of narrow molecular weight distribution standards and plotting the log of their peak molecular weight versus their retention volume. If the curve is linear, then it obeys:

$$\log M = A + B v \tag{1}$$

However, even with the new "linear" columns, some curvature is evident and the data is fit by a cubic polynomial (e.g., see refs. 1–3):

$$\log M = A + B v + C v^2 + D v^3 \tag{2}$$

Also, the intrinsic viscosity of each standard versus retention volume can be similarly fit as can the hydrodynamic volume versus retention volume.

In this investigation, we are interested in knowing if the fractionation attained by the columns is, in practice, well represented by such equations.

To examine this, a plot of the difference between the fitted equation and experimental values of log M can be plotted versus retention volume. This is termed a "plot of residuals" where:

$$\text{residual} = (\log M)_{\text{fit}} - (\log M)_{\text{std}} \tag{3}$$

A non–random pattern of data indicates that the data is not well fit by the equation. A magnitude of scatter in excess of what is desired indicates poor SEC reproducibility. However, the practical utility of this type of plot is limited because of our inability to specify what is a meaningful magnitude of scatter on the log M axis.

Most often the chromatograms are used as a source of molecular weight averages. They can be calculated from the refractometer chromatogram from:

$$M_k = \frac{\int w(v)\,(M(v))^{k-1}dv}{\int w(v)\,(M(v))^{k-2}dv} \tag{4}$$

where $k = 1$ for M_n, 2 for M_w and 3 for M_z. Thus, the averages are weighted sums over the chromatogram with $1/M$, M or M^2 as the weighting factors. It should be noted that log M does not enter the calculation of the averages.

Therefore, a more useful plot of residuals is to utilize molecular weight rather than log M. Furthermore, if we use percent error in molecular weight rather than only the deviation in molecular weight we retain a link to the fact that we expect the percent random error in the specified molecular weight of standards to be a constant. Then, the definition of residual that was used in plots of residuals is:

$$\text{Residual (\% Error in M)} = \frac{100(M_{fit} - M_{std})}{M_{std}} \qquad (5)$$

Residuals for intrinsic viscosity are similarly defined:

$$\text{Residual (\% Error in } [\eta]) = \frac{(100[\eta]_{fit} - [\eta]_{std})}{[\eta]_{std}}$$

The Use of Molecular Weight Detectors

The prime difficulty in using the conventional calibration curve as a basis for assessing SEC columns is that it is affected by many variables in addition to the fractionation characteristics of the columns. Concentration of injected standards, flow rate fluctuations, polydispersity of narrow standards, temperature, and inaccurate vendor assigned values for the averages of the standards can all contribute to the result. The last mentioned variable is particularly troublesome. Vendor molecular weight averages have been found to be as much as 20% in error (4–6).

The low angle laser light scattering (LALLS) and differential viscometry (DV) detectors can be used to provide a direct measure of the "whole polymer" weight average molecular weight, M_w, and intrinsic viscosity, $[\eta]$, respectively. Thus, they provide alternative estimates to those of the vendor. Furthermore, when both of these detectors are used as part of the same SEC system together with the refractometer, all the estimates are obtained simultaneously.

These "whole polymer" values are calculated from LALLS and DV using the following equations for LALLS:

$$M_w = \frac{\int R_\theta(v)dv}{\int c(v)dv} \qquad (6)$$

where $R_\theta(v)$ is excess Rayleigh scattering at retention volume v, and for DV:

$$[\eta] = \frac{\int \eta_{sp}(v)dv}{\int c(v)dv} \qquad (7)$$

where η_{sp} is specific viscosity.

LALLS and DV are subject to various sources of error. However, by using equations 7 and 8 in place of the usual summation equations (e.g., equation 4) we can expect to maximize precision. Furthermore, when calculated by equations 7 and 8, M_w and $[\eta]$ are not affected by chromatographic resolution (axial dispersion) for these two respective detectors.

Alternative Representations of Calibration Curves

If a simple polynomial does not fit the calibration data, there are several alternatives available:

1. Other equations may be used. The Yau Malone Equation (7) is an example of a curve used in the SEC literature.
2. A polynomial can be fit to only the range of retention volumes represented by the unknown to be analyzed.
3. Splines can be used. This means fitting consecutive segments of the calibration curve data with attention to continuity of the curve at the segment boundaries. Special attention is required to prevent the spline equations from overfitting the data (i.e., snaking through each and every data point).
4. Moving average methods of smoothing and interpolating can be tried.

EXPERIMENTAL

Three 7.5 mm i.d. x 300 mm 5-μm particle diameter PL Gel mixed-bed columns (Polymer Laboratories, Amherst MA) were coupled in series. Uninhibited HPLC-grade tetrahydrofuran (THF) (J. T. Baker) was filtered through a 0.2 μm PTFE filter, vacuum degassed and continuously sparged with helium. THF eluent was delivered at a nominal flow rate of 1.0 mL/min by a Waters Associates (Milford, MA) Model 590 reciprocating piston pump equipped with a high sensitivity pulse-damping coil. The column effluent was split nearly equally to a Model 100 differential viscometer (Viscotek Corporation, Porter, TX) and a LALLS photometer (LDC/Milton Roy, Riviera Beach, FLA) as shown in Fig. 1. A Waters Associates Model 410 differential refractometer thermostated to 35°C was connected in series to the LALLS photometer. The columns, splitter tee, pulse damping coil made from 10 ft of 0.04 in. i.d., 1/16 in. o.d. stainless steel tubing capped at one end and the differential viscometer were thermostated to 30.0 ± 0.1°C. The volumetric flow between the detectors was equalized by back pressure on the viscometer. The split flow stream was recombined and passed through a thermal pulse flowmeter (Molytek Corporation, Pittsburgh, PA). This combined parallel/serial configuration provides less axial dispersion in the DRI detector and more evenly balances extra-column broadening among the detectors than the serial arrangements employed by other workers (8,9). Narrow standard polystyrenes and poly(methyl methacrylates) (American Polymer Standards, Mentor, OH) above 800,000 daltons were injected individually at a concentration of 0.1 mg/mL in a volume of 100 μL. Polymer standards below 800,000 daltons were injected individually at progressively higher concentrations

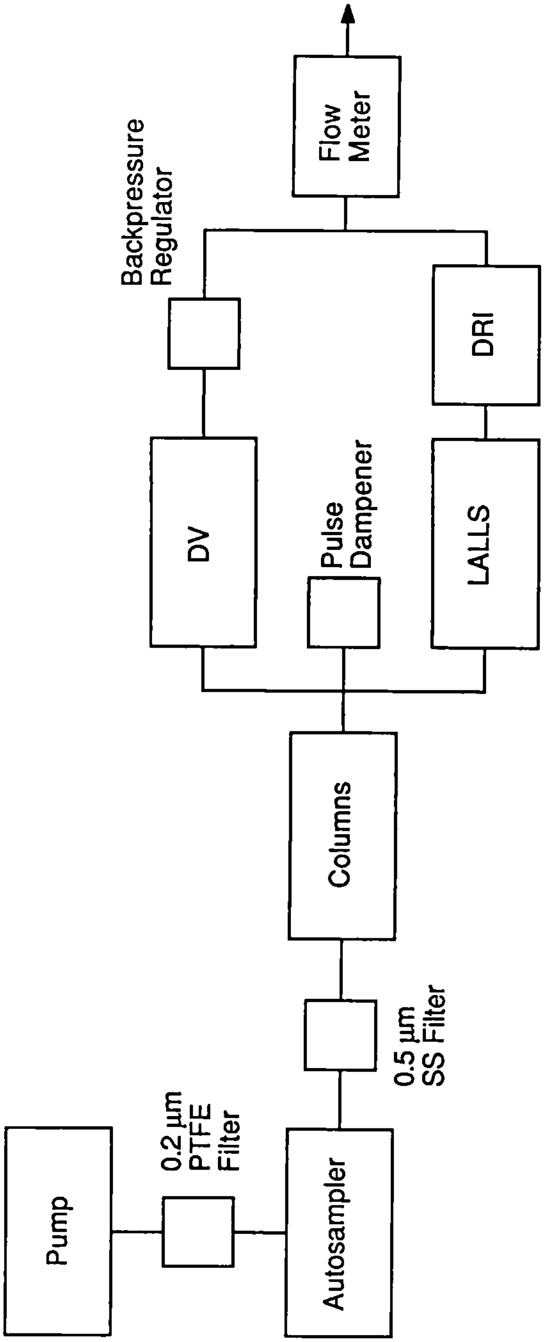

FIGURE 1. LALLS/DV/DRI detector schematic.

up to 1.0 mg/mL for the lowest molecular weight standards. Broad-molecular-weight-distribution polymers were injected at a concentration of 2.0 mg/mL in a volume of 100 µL. The differential pressure transducer of the viscometer was calibrated with several narrow and broad molecular weight distribution polystyrenes of known intrinsic viscosities. The thermal pulse flow meter was calibrated by collecting 5-mL aliquots in a gravimetrically calibrated volumetric flask. LALLS attenuators were calibrated as described by the manufacturer, and experiments were not performed unless Rayleigh factors for pure THF were within 10% of the literature value for 632.8 nm incident radiation. Refractive index increments used in LALLS calculations were 0.184 for polystyrene and 0.084 for poly(methyl methacrylate). Detector lag volumes were measured without columns at a flow rate of 0.3 mL/min by injecting an organic compound which absorbs 632.8 nm radiation, as described elsewhere (10). Data from the three detectors and the thermal pulse flow meter were collected simultaneously using Viscotek version 1.0 LALLS and version 3.0 UNICAL software which had data processing routines modified in-house. An average flow rate for each sample was calculated from the output of the flowmeter.

RESULTS AND DISCUSSION

Assessment of Conventional Calibration Curves

Narrow standard calibration curves for polystyrene and poly(methyl methacrylate) plotted as log M versus v are shown in Figs. 2 and 3. Figures 4 and 5 show log [η] versus retention volume. The fit of a cubic polynomial is displayed in each figure. Also plotted are data points corresponding to values measured by LALLS and DV. For samples below 10,000 daltons, LALLS signals were too weak at the concentrations examined to calculate M_w. All of these plots appear normal with good agreement of data and fitted lines throughout.

Figures 6 to 9 show plots of residuals (equation 5) versus retention volume corresponding to the calibration curves of Figs. 2 through 5. Now the situation does not appear "normal". A definite "winding" of the data around the fitted line is clearly evident in Figs. 6 to 9. Furthermore, the deviation of the points from the fitted line is often about 10% when expressed as % error in molecular weight. Vendor values are consistently higher than either LALLS or DV values. However, both types of data show the consistent winding pattern. In this work, the precision of M_w values from LALLS as determined by measurement on four samples of polystyrene 600,000 (each examined in duplicate LALLS runs) was ±1.3% relative standard deviation. Whole polymer intrinsic viscosity was determined by DV to ±1.1%.

Precision of this order was obtained for standards between molecular weights 10,000 and 1,000,000 and is clearly smaller than the magnitude of residuals in Figs. 6 and 8. With standards beyond this range poor signal-to-noise ratio on one or more detectors decreased the precision attainable.

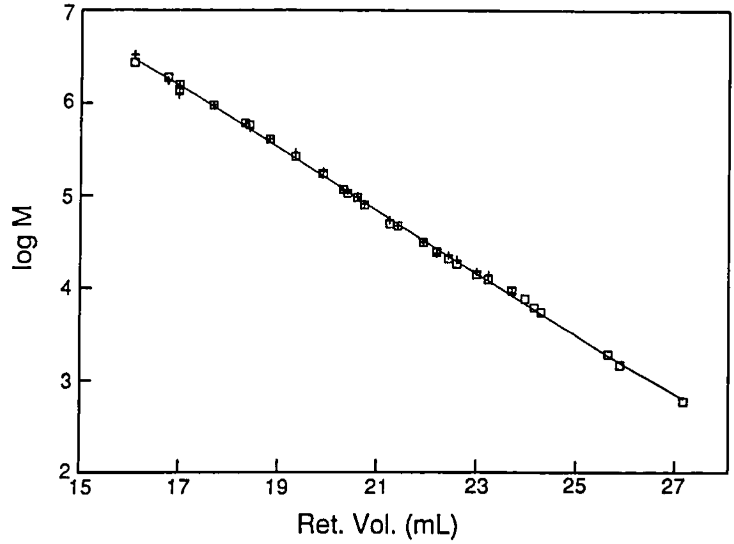

FIGURE 2. Conventional polystyrene narrow standard calibration. □ Vendor Mp values; + LALLS M_w.

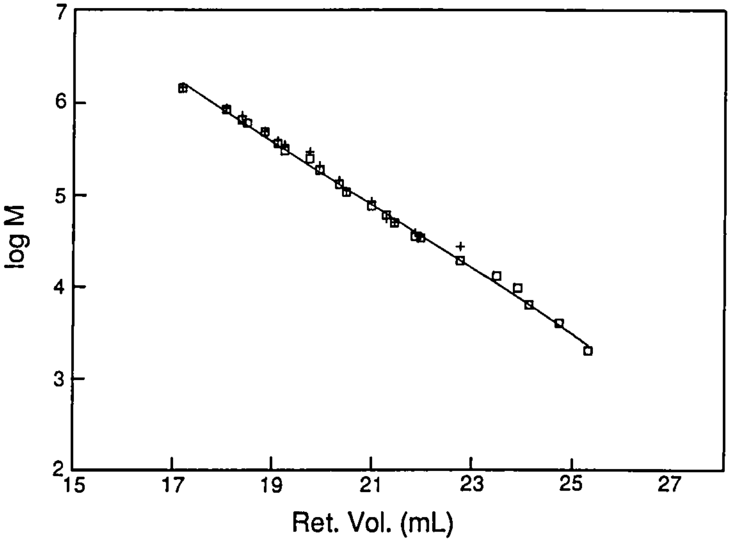

FIGURE 3. Conventional poly(methyl methacrylate) narrow standard calibration. □ Vendor Mp values; + LALLS M_w.

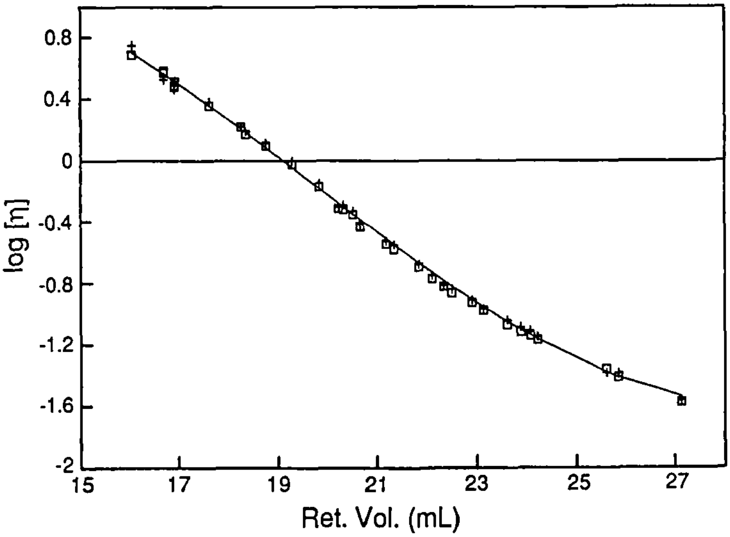

FIGURE 4. Best third-order polynomial fit of narrow standard polystyrene intrinsic viscosities. □ Vendor [η] values; + measured by differential viscometry.

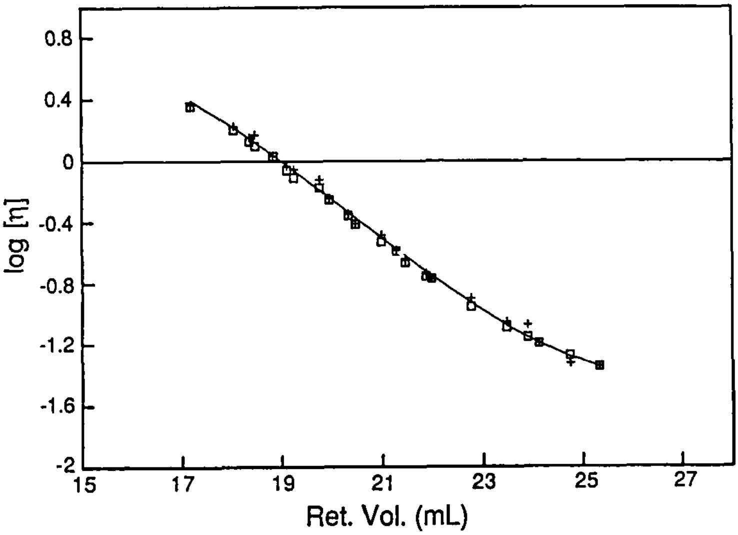

FIGURE 5. Best third-order polynomial fit of narrow standard poly(methyl methacrylates). □ Vendor [η] values; + measured by differential viscometry.

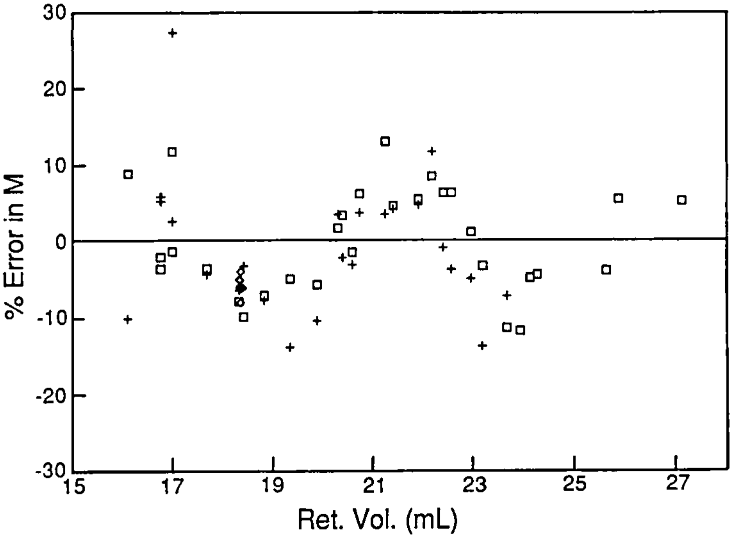

FIGURE 6. Percent error in M of polystyrene narrow standards. □ Vendor Mp; + LALLS M_w; ◇ PS600,000 replicates.

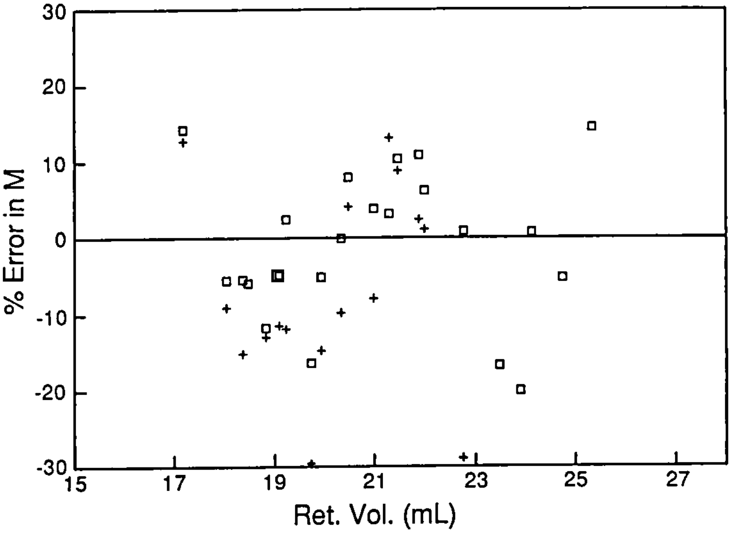

FIGURE 7. Percent error in M of poly(methyl methacrylate) narrow standards. □ Vendor Mp; + LALLS M_w.

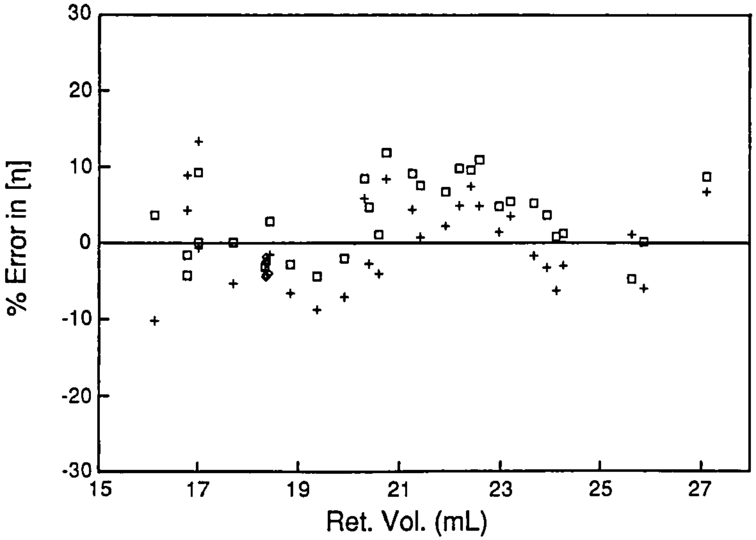

FIGURE 8. Percent error in [η] of polystyrene narrow standards. □ Vendor Mp; + measured by differential viscometry; ◇ PS600,000 replicates.

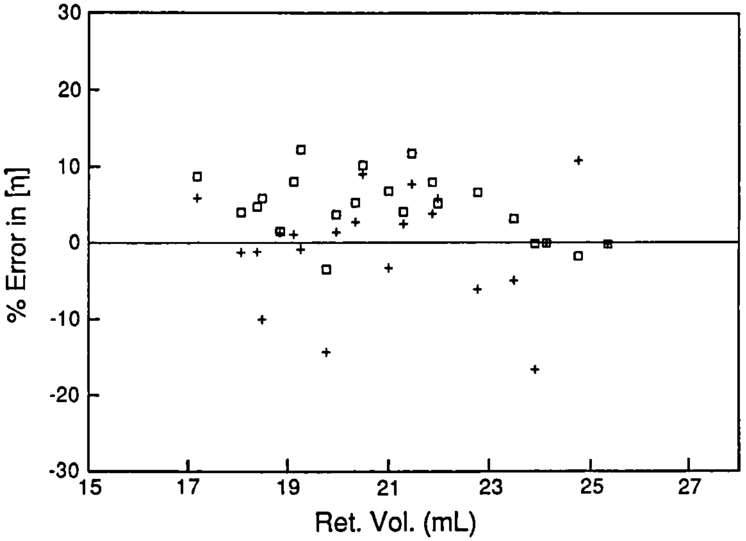

FIGURE 9. Percent error in [η] of poly(methyl methacrylate) narrow standards. □ Vendor [η]; + measured by differential viscometry.

The precision of retention volume measurements, corrected for flow fluctuations via use of flowrates measured by the thermal pulse flow meter in this experiment is ±0.1% relative standard deviation across the elution volume range examined. This compares favorably with precision quoted by other users of this flowmeter (11, 12). Uncertainty in the retention values are an order of magnitude lower than uncertainty in the molecular weight values. This is an implicit assumption in the use of linear regression for the size exclusion calibration curve and the derived plot of residuals.

Considering the above results, it was evident that the simple, third–order polynomial was inadequate to describe the complex irregularities in the calibration curve. Furthermore, these irregularities apparently originated with the column packing. They could be due to imperfect overlap regions of packing particles with different pore sizes.

Accounting for Calibration Curve Irregularities

In this work the only alternative examined was fitting of a polynomial to the calibration data over the range of elution of an unknown. This is easy to implement since most commercially available SEC software packages include cubic polynomial fit routines. Figure 10 shows the results when NBS 706 polystyrene was analyzed using the data of Fig. 2 using the polynomial first for all of the calibration data and then for only the region from 17 to 24 mL. Table 1 compares molecular weight averages. The best values of NBS 706 are presumed to be the most recently obtained by Alfredson and Haney. Figure 11 shows the plot of residuals for the abbreviated fit to the calibration curve. The magnitude of the residuals is now comparable to the uncertainty in the molecular weight values and the residuals are more randomly scattered about zero. The significant change in the molecular weight distribution, the improvement in the molecular weight averages and the removal of trends from the plot of residuals all show that this method did account for the curve irregularities. Furthermore, the results show that the correction was very worthwhile.

Assessment of Universal Calibration Curves

The analysis described in the previous two sections for conventional molecular weight and intrinsic viscosity calibration curves was then extended to the universal calibration curve. Figure 12 shows the calibration curve. Figure 13 shows a plot of residuals. Similar trends to those previously observed were evident. This time, deviations from the fitted line are often approximately 20%. Figure 14 and Table 1 shows the results on the molecular weight distribution and molecular weight averages of utilizing an abbreviated calibration curve. Again, the results with the conventional calibration curves were echoed. By comparing Figs. 14 and 10 it can be seen that the molecular weight distributions calculated by universal calibration are more susceptible to errors caused by inappropriate fitting of calibration data than distributions calculated by conventional narrow standard calibration.

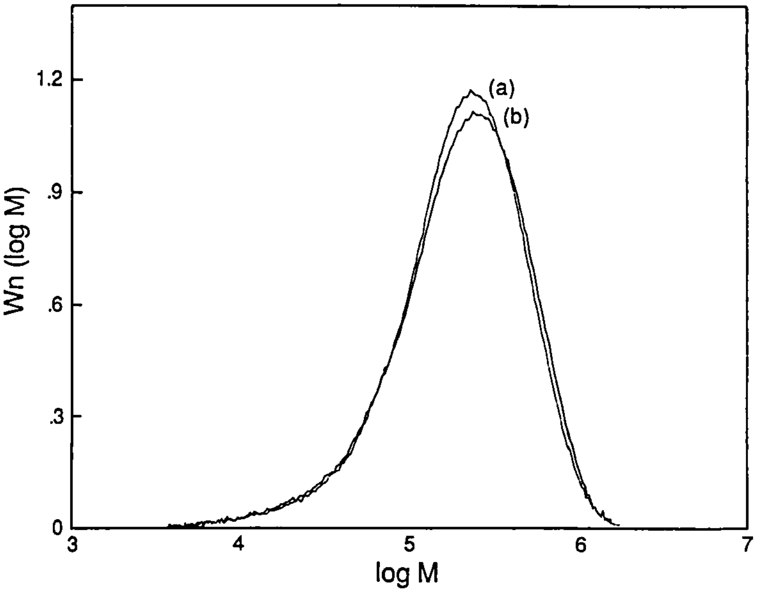

FIGURE 10. NBS 706 molecular weight distributions calculated by conventional calibration using third-order fit of (a) all polystyrene calibration data, (b) calibration data between 17 and 24 mL.

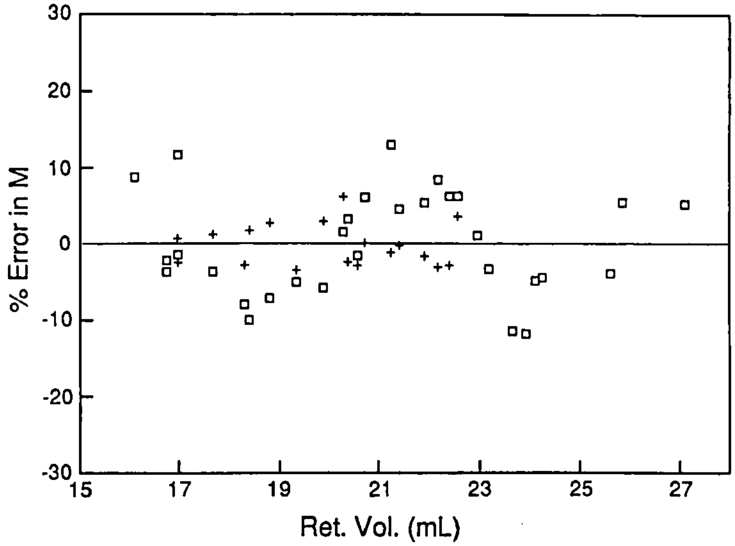

FIGURE 11. Percent error in M of polystyrene narrow standards for third-order fits of □ all calibration standards; + standards eluting between 17 and 24 mL.

TABLE 1

NBS 706 Polystyrene

This Study	M_n	M_w	M_z
Conventional Calibration			
all stds 3rd order fit	126000 ± 3600	265000 ± 2600	427000 ± 3700
abbreviated 3rd order fit	119000 ± 3400	274000 ± 2700	444000 ± 3800
Universal Calibration			
all stds 3rd order fit	132000 ± 10600	260000 ± 8900	416000 ± 13800
abreviated 3rd order fit	123000 ± 10400	277000 ± 9000	451000 ± 14800
LALLS	117000 ± 11500	277000 ± 10500	455000 ± 16000
Literature			
Alfredson (13)[a]	123000	276000	-
Haney (14)[a]	127000	272000	460000
Kato (15)[b]	137000	272000	-
NBS	136500[c]	257800[d] 288100[f]	395900[e]

(a) Analytical SEC
(b) Preparative SEC
(c) Membrane osmometry
(d) Light scattering
(e) Viscometric analysis of fractions
(f) Sedimentation equilibrium

Use of a Polydisperse Standard to Elucidate Calibration Irregularities

Assessment of columns by the methods outlined above requires the injection of many narrow standard polymers. In an effort to reduce this workload a mixture of broad poly(methyl methacrylate) standards was injected. The objective was to see if the elution profile of a high resolution SEC column set could be characterized with only one polydisperse sample from the directly measured local property values available from the LALLS and DV. Results are shown in Figs. 15 to 18. Four data files from independent runs were averaged to generate these plots. Figures 15 and 16 show calibration curves obtained from the DV and LALLS, respectively, used on the single broad PMMA standard superimposed on the conventional calibration curves obtained from the injection of narrow standards. The curves in each figure are expected to superimpose. The fact that they do not can be due to many variables. Flow rate variations and inter–detector volume are known to strongly affect these plots. Other variables include solvent refractive index, polymer refractive index increment, attenuator filter factors, and mis–matched sensitivity at the tails of the chromatograms on the different detectors. The oscillations at the tails of the curves in Figs. 15 and 16 are attributed to this latter

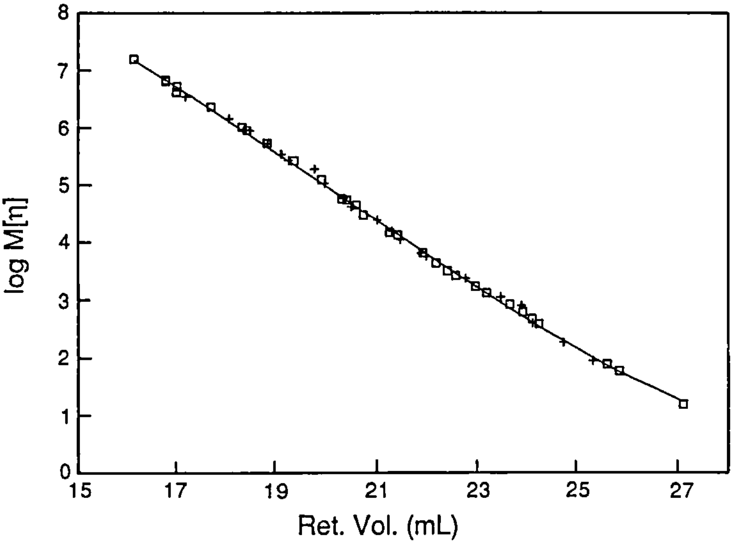

FIGURE 12. Universal calibration plot. □ Polystyrene; + poly(methyl methacrylate).

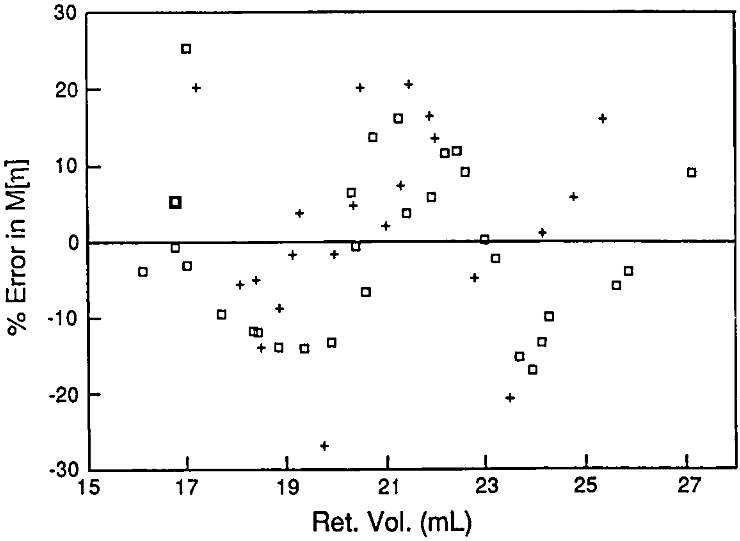

FIGURE 13. Percent error in M[η] for universal calibration plot. □ Polystyrene; + poly(methyl methacrylate).

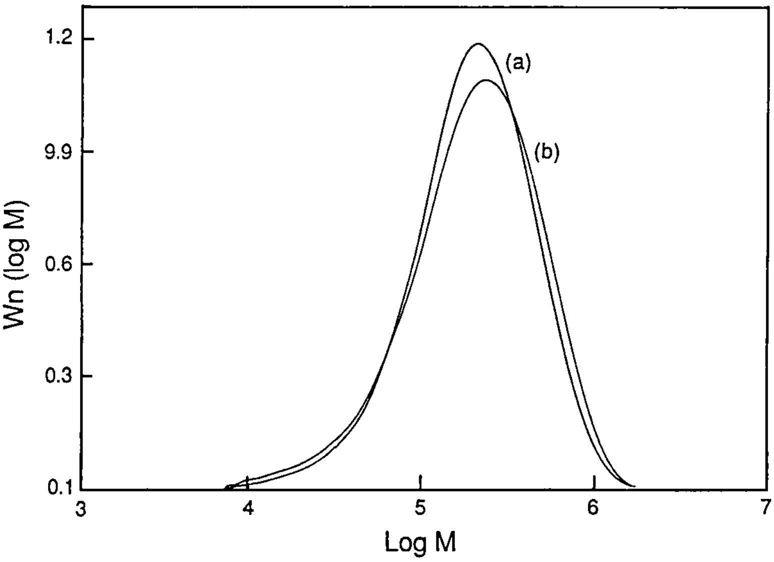

FIGURE 14. NBS 706 molecular weight distributions calculated by universal calibration using third-order fit of (a) all polystyrene calibration data; (b) calibration data between 17 and 24 mL.

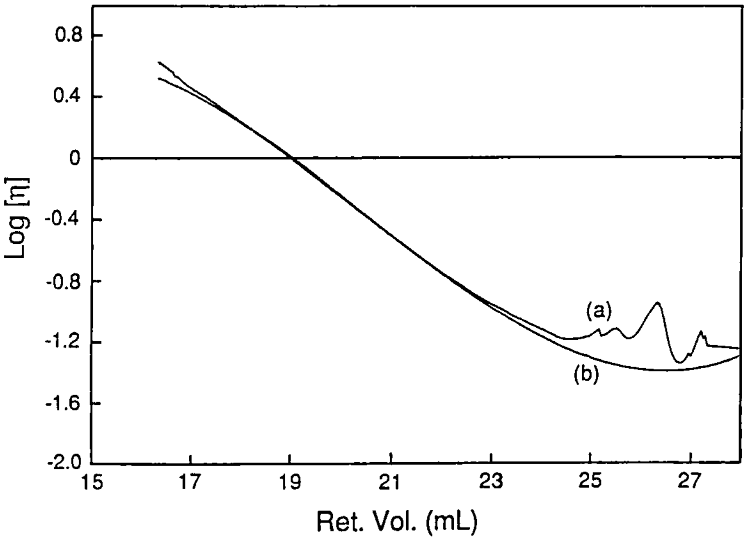

FIGURE 15. Broad mixture of poly(methyl methacrylates). Output of (a) differential viscometer; (b) best third-order polynomial fit of narrow standard poly(methyl methacrylate) data of Fig. 5.

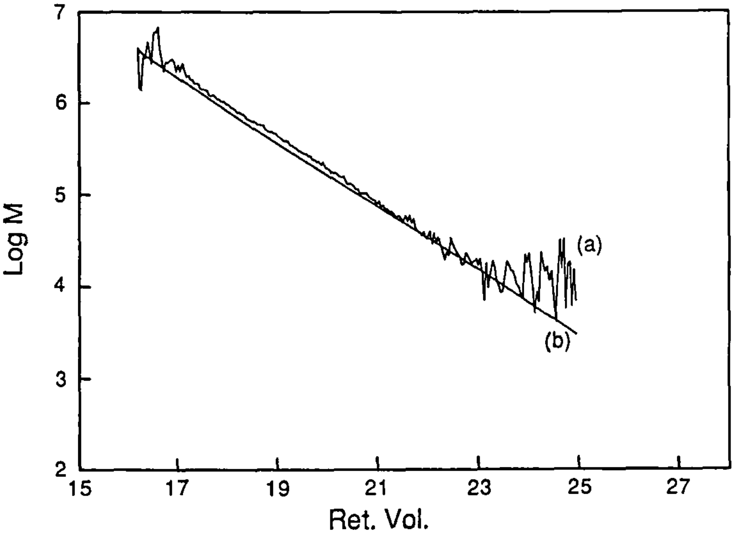

FIGURE 16. Broad mixture of poly(methyl methacrylates). Output of (a) LALLS; (b) best third-order fit of narrow standard poly(methyl methacrylate) data of Fig. 3.

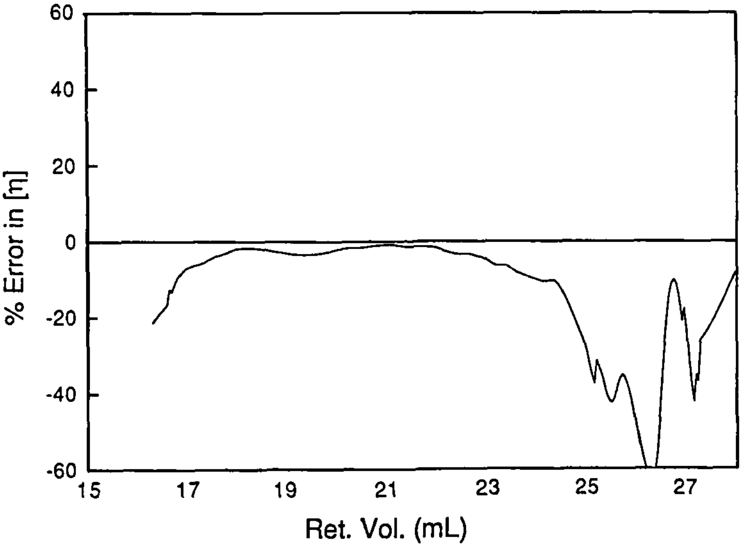

FIGURE 17. Percent error in [η] measured by differential viscometry detector of broad mixture of poly(methylmethacrylates).

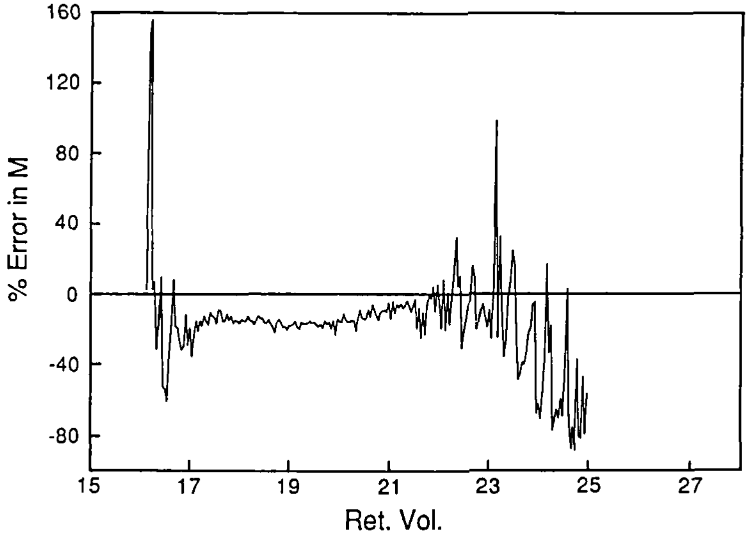

FIGURE 18. Percent error in M measured by LALLS detector of broad mixture of poly(methyl methacrylates).

reason. They occurred despite the averaging of the four standards to better define these tails. Axial dispersion effects may be causing some of the differences. However, both theoretical and experimental results in the literature indicate a low contribution from this cause.

Figures 17 and 18 show plots of residuals using the points derived from the single injected broad standard and the cubic equation fitted to the conventional calibration curve. The sources of error discussed in the preceding paragraph completely masked any trends of the calibration points about the polynomial. Further development directed at using a single polydisperse standard alone (with no conventional calibration curve) was therefore not attempted.

CONCLUSIONS

o High resolution SEC columns can show irregular fractionation characteristics (16). These characteristics become evident as data points winding about the fitted line when a plot of residuals is derived from the conventional calibration curve and expressed in terms of the percent error in molecular weight.

o The deviation of the calibration data from the fitted line was approximately 10% for the conventional molecular weight and intrinsic viscosity calibration curves. It became 20% for the universal calibration curve.

○ LALLS and DV detectors were used together with the DRI detector to provide evidence that the calibration curve deviations were due to the column packings and not due to some other cause (e.g., vendor values of molecular weights and intrinsic viscosities).

○ Use of a polynomial fit to a portion of the curve corresponding to the retention volume range of an unknown was used to show the significant improvement in results which occurred when the calibration variations were taken into account.

○ At present, use of many individual narrow standards is necessary to elucidate the effect. The effect was masked when a single polydisperse standard was used because results were affected by a multitude of variables.

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