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NON-LINEAR CALIBRATION BY CUBIC SPLINES IN GEL PERMEATION CHROMATOGRAPHY

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SUMMARY

A general method for calibration in gel permeation chromatography is proposed, based on a least squares approximation of the calibration curve by cubic splines using polymer standard(s) with one or more known molecular weight averages. The calibration using polymer standards having broad molecular weight distributions with known M_n and/or M_w is discussed and compared with similar methods.

The effectiveness of cubic splines for the approximation of the calibration curve is a consequence of the inherent local properties and smoothness of the model. The natural cubic spline model with equal intervals is the preferred choice for non-linear calibration. The linear calibration curve is obtained as a special case when information is sparse.

INTRODUCTION

In order to determine the molecular weight distribution of polymers by gel permeation chromatography (GPC), one must relate the molecular weight to the elution volume, since the differential weight distribution obtained experimentally is a function of the volume. The calibration method for determining this relation is normally based on the GPC measurements of a number of polymer standards having one or more known molecular averages. Assuming that a calibration curve between molecular weight and elution volume can be uniquely defined for the standard(s) used, this curve could be approximated by a mathematical function, $M(v)$, according to a least squares procedure. The values of the parameters, c , in the model are estimated by minimizing the weighted square of the deviations between the observed (GPC determined) averages, $M_{i,k}$, and the expected (absolutely determined) averages, $M_{i,k}^0$, for the molecular weight averages i of standard k :

$$X^2 = \sum_{k=1}^n \sum_{i=1}^m (M_{i,k} - M_{i,k}^0)^2 / M_{i,k}^{0,2}$$

However, since the sum of the squares could be made arbitrarily low by increasing the number of parameters, a low X^2 value is not a sufficient criterion for

selecting the appropriate number of parameters in the model or for comparing different models proposed for the calibration curve. Furthermore, the possibility of unequal reliability of the different absolute methods for measuring the known molecular weight averages has not been taken into account.

For an evaluation of cubic splines as an approximation of the calibration curve, a more general procedure for the least squares approximation has therefore been adopted. The estimate of the parameters, c , which minimizes the reduced X^2 value

$$X^2 = 1/(nm - q) \sum_{k=1}^n \sum_{i=1}^m (M_{i,k} - M_{i,k}^0)^2 / s_i^2$$

is accepted, where nm is the total number of averages used and q the number of parameters. The weighting factors, s_i^2 , are equal to the sum of the variances for the GPC determined and absolute molecular weight averages, i . Assuming that any systematic error has properly been accounted for, X^2 should be approximately unity if a large number of standards with known molecular weight averages are available. In practice, however, the number of available polymer standards with known molecular weight averages are always limited, and the number of parameters, q , is then chosen so as to minimize X^2 . For cases where the variances of the observed and expected averages are known only approximately or not at all, the weighting factors should be equal to $M_{i,k}^0{}^2$ to provide equal relative weight over the calibration range.

Calibration curve models

A theoretical model based on diffusion has been derived by Yau and Malone¹ for the relation between the molecular weight and the elution volume:

$$v = c_1 + c_2 \{1/\sqrt{\pi} \psi [1 - \exp(-\psi^2)] + \operatorname{erfc}(\psi)\}$$

where

$$\psi = M^{c_4}/c_3$$

This equation has been used for calibration with standards having both narrow and broad molecular weight distributions^{2,3}. The unique advantage of this model is the connection between the parameters and the separation process, where c_1 represents the exclusion volume, v_0 , and $c_1 + c_2$ the total volume, v_t . If one has a sufficient number of well-defined polymer standards covering the calibration range of the packing material, this physical significance of the parameters should be confirmed if the model is an adequate representation of the calibration curve.

For cases where there are only a small number of well-characterized standards or where the GPC instrument employs a series of columns packed with materials of different pore sizes, the theoretical model may be unsatisfactory and empirical models often are appropriate. A common example is the fitting of a polynomial to experimental data in order to predict the separation response as a function of two or more variables⁴⁻¹¹.

A modified polynomial model for the calibration curve has also been proposed

$$M = c_5 + \exp(c_1 + c_2 v + c_3 v^2 + c_4 v^3)$$

and tested with standards having broad molecular weight distributions¹².

As the calibration graph has a slightly curved part surrounded by a sharp rise and a sharp decrease, it is in general not suited for approximation by a single polynomial. The polynomial representation is more or less limited to the linear region. The polynomial fitting is also complicated by strong interaction between parameters, which leads to a progressively poorer least squares approximation as the number of parameters increases. A more appropriate function is then the orthogonal polynomial which, while mathematically equivalent to the common polynomial, is unaffected by such interactions. A suitable choice is the Legendre polynomial, which is orthogonal with a weighting factor of unity over the closed interval $-1 \leq x \leq 1$. The coordinate range must therefore be defined in accordance with this interval and the calibration curve approximated by the function

$$\ln M = \sum_{i=1}^q c_i P_i(x)$$

where

$$x = (2v - v_0 - v_1)/(v_1 - v_0)$$

and $P_i(x)$ is the Legendre polynomial.

Linear fitting is most often applied to calibration curves since the logarithm of the molecular weight of standards having sufficiently narrow distributions yields an almost linear calibration curve *versus* peak elution volume for certain column sets. As an alternative to the use of higher-order polynomials for non-linear calibration, the approximation of a non-linear calibration curve by straightline segments has been proposed⁴. However, since this approach will not give continuous differential weight distributions, it is of questionable value for non-linear calibration. The treatment of curves as composed of segments of straight lines can nevertheless be extended to represent curves composed of segments of polynomials of an arbitrary degree. The most useful of these are the cubic splines, defined according to Ahlberg *et al.*¹³.

For an interval $v_0 < x < v_1$, subdivided by a mesh

$$\Delta: v_0 = x_0 < x_1 < \dots < x_n = v_1$$

an associated set of ordinates is prescribed:

$$Y: y_0, y_1, \dots, y_n$$

Then the function $S_\Delta(x)$, which is continuous together with its first and second derivatives on v_0 to v_1 , coincides with a cubic in each subinterval $x_{j-1} < x < x_j$ ($j = 1, 2, \dots, N$) and satisfies $S_\Delta(x) = y_j$ ($j = 0, 1, \dots, N$), is said to be a spline with respect to the mesh Δ . For a cubic spline defined in this way there are $4N$ defining

constants: requiring derivatives of orders 0, 1, 2 to be continuous at each interior mesh point accounts for 3 ($N - 1$) degrees of freedom, leaving $N + 1$ to interpolation at the mesh points and two end conditions at $x = x_0$ and $x = x_N$. If the end conditions are chosen such that the second derivative is equal to zero at $x = x_0$ and $x = x_N$, a natural cubic spline is obtained. Among all the cubic splines that interpolate the prescribed ordinates, only the natural cubic spline minimizes the integral:

$$\int_{x_0}^{x_N} S''(x)^2 dx$$

The natural cubic spline thus minimizes curvature and gives a smooth representation of the calibration curve. For $N = 1$ it reduces to a linear representation of the calibration curve, and the choice of end conditions is based on the fact that this curve represents the natural choice when the information is limited, as is also the case for the ends of the calibration curve.

The unique advantages of the cubic spline model for the approximation of the calibration curve are due to the smoothness and local properties of the model. Even if the spline is a global curve in the sense that alteration of a single ordinate affects the spline throughout the interval from x_0 to x_n , the effect declines rapidly as the distance from the altered ordinate increases. From a numerical viewpoint, this means that if one chooses the ordinates as parameters for the estimation, the parameters are nearly independent of each other. A further consequence of the local property is that the cubic spline model is a better approximation of the calibration curve for a general multicolumn set, where the calibration curve comprises contributions from the different packing sizes, each covering a separate range of the calibration curve. Also, the smoothness of the spline function gives it a significant advantage over higher order polynomials when dealing with sparse data. The term sparse is here loosely defined as meaning that the number of parameters is more or less equal to the number of known molecular weight averages. In this case the cubic spline model is less vulnerable to the wiggles and oscillations of a higher-order polynomial, due to the sacrificing of continuity of higher derivatives for the smoothness of the second derivative.

EXPERIMENTAL

Standards

Seven well-characterized broad dextran standards (Pharmacia, Uppsala, Sweden) were used for the calibration. The M_n values of these standards were determined by end-group analysis by the Somogyi copper phosphate method and M_w values by light-scattering measurements^{1,2}.

GPC procedure

The GPC measurements were performed with a glass column (1000 × 6.3 mm I.D.) slurry packed with Hydrogel VI (particle size < 37 μm) (Waters Assoc.). The average plate count during the runs was 6000 plates per m as measured with methanol at a flow-rate of 6 ml/h (20 ml/cm² · h).

The 0.25-ml degassed samples contained 2 mg/ml of the dextran standards and 1 μl/ml of methanol in dilute H₂SO₄, pH = 3. The methanol was added as an internal standard.

Calibration curve models

Six models were used:

(I)	Theoretical model based on diffusion	(Diffusion model)
(II)	Legendre polynomials	(Legendre model)
(III)	Polynomials	(Polynomial I model)
(IV)	Modified polynomial model	(Polynomial II model)
(V)	Natural cubic spline model	(Spline I model)
(VI)	Cubic spline model	(Spline II model)

Numerical methods

The computer programs for the evaluation of the calibration curve models were written in Fortran IV. To simplify them for the non-linear least squares estimation, a standard finite difference Levenberg–Marquardt subroutine ZXSSQ supplied by IMSL (International Mathematical and Statistical Libraries, Houston, TX, U.S.A.) was used. The subroutines for the cubic spline interpolation and evaluation were also based on the IMSL subroutines ICSICU and ICSEVU. Since these IMSL subroutines are standard programs, any similar subroutines from a larger library of subroutines could probably be used instead.

RESULTS AND DISCUSSION

During the preliminary runs it became evident that distilled water was unsatisfactory as eluent since some of the dextran standards gave a high-molecular-weight fraction. Since the packing material as well as the dextran standards contains a small number of ionic sites (presumably carboxylate groups judging from the information from the manufacturers), an ionic exclusion effect is probably responsible for these distributions. The normal procedure for obviating such an effect is to use an eluent with a sufficiently high ionic strength. Since a salt solution with an ionic strength of >0.02 also gave small salt peaks at the total volume, an eluent of $0.5 \text{ mM H}_2\text{SO}_4$ was used, which gave chromatograms identical to those obtained by the normal procedure, except for the absence of any salt peak at the total volume. The successful use of this eluent at $\text{pH} = 3$ is due to the fact that the carboxylate groups are almost uncharged at this pH.

As an estimate of the precision of the absolute methods, data have been taken from Table I in ref. 12. From this table it can be concluded that, whereas the relative standard deviation is approximately independent of the molecular weight, a discrepancy is observed between the two absolute methods. A pooled value of the variance for each method can then be obtained from

$$s_i^2 = \left(\sum_{j=1}^n p_j - n \right)^{-1} \sum_{j=1}^n (p_j - 1) s_{j,i}^2$$

where p_j is the number of replicates in a sample and n the number of samples. Excluding the first entry in the table, as this standard is outside the range of the commercially available dextran standards, a relative standard deviation of 4.8% for M_n and 2.9% for M_w is obtained.

Three series of the standards were run in random order in each series, so that

the variance of the GPC measurements could be evaluated. The chromatograms were compensated for any flow-rate variation from run to run by the internal methanol standard. From the results given in Table I the variation of the relative standard deviation with the molecular weight seems essentially random, and an estimated relative standard deviation of 1.2% for M_n and 1.0% for M_w can be obtained from the mean value of the variances. In comparison with the values of the relative standard deviations for the absolute methods of 4.8% for M_n and 2.9% for M_w , the GPC contribution to the total variance is small and constitutes only 6% and 11% respectively. The calibration curve models can therefore be evaluated by one of the three series of GPC measurements, since the deviations between the measured and known molecular weight averages are approximately determined by the random uncertainty in the measurements of the known averages. In general, however, this may not be the case, and it is then essential to take this contribution into account in the calibration.

TABLE I

RELATIVE STANDARD DEVIATIONS (%) OF MOLECULAR WEIGHT AVERAGES AS OBTAINED FOR DEXTRAN STANDARDS FROM THREE MEASUREMENTS

	<i>Standard*</i>						
	<i>T 10</i>	<i>T 20</i>	<i>T 40</i>	<i>T 70</i>	<i>T 110</i>	<i>T 150</i>	<i>T 250</i>
$s(M_n)$	0.71	1.41	0.60	1.48	1.70	0.15	1.69
$s(M_w)$	0.49	1.09	0.39	0.59	1.66	0.47	1.58

* The number in the code for each standard refers to approximately $M_w/1000$.

The results of the calibration with known M_n and/or M_w for the different calibration curve models are given in Table II. Since the basic assumption of the GPC separation process is that the molecules are eluted in the inverse order of their hydrodynamic volume or the molecular weight for homopolymers, the calibration curve model must have a first derivative less than zero to be an acceptable representation of the calibration curve. When the number of parameters in the models increased this condition was not met, and these solutions must therefore be excluded. For the numerical method the main requirements of the models for non-linear estimation are efficient convergence, minimal parameter interaction and easy choice of the initial guesses for the parameters.

The natural cubic spline model (Spline I model) could be considered as fulfilling all these requirements. In all runs, the non-linear least squares fit could be computed in single precision* with a minimum number of functional evaluations. This effectiveness is a consequence of the inherent advantage of the cubic spline method for the approximation and the choice of the ordinates as parameters. When the ordinates are uniformly located over the elution volume range, minimal parameter interaction is obtained. Since the values of the ordinates are of the same magnitude, no scaling error will occur, and the number of significant digits is approximately the

* For an IBM computer, "single precision" is 4 bytes of storage or approximately 7.2 decimal digits for a variable; "double precision" is 8 bytes of storage for a variable.

TABLE II

X^2 VALUES FOR THE CALIBRATION CURVE MODELS WITH CALIBRATION BASED ON M_n AND/OR M_w

The optimal least squares fits were obtained with four parameters and are indicated by the italicized X^2 values.

M_n	M_w	q	<i>Spline I</i>	<i>Spline II</i>	<i>Polynomial I</i>	<i>Legendre</i>	<i>Polynomial II</i>	<i>Diffusion</i>
X	X	2	7.166	7.166	7.166	7.166		
X	X	3	5.020	4.611	4.611*	4.611		
X	X	4	0.953	0.796	0.796*	0.796		0.747
X	X	5	0.957	0.883	0.884*	0.878*	0.883*	
X	—	2	1.591	1.591	1.591	1.591		
X	—	3	1.173	1.185	1.185*	1.185		
X	—	4	0.823	0.850	0.850*	0.850*		0.888
X	—	5	1.164	1.168*	1.276*	1.162***	1.114***	
—	X	2	14.29	14.29	14.29	14.29		
—	X	3	4.823	3.762*	3.762*	3.762		
—	X	4	2.195	1.762*	1.762*	1.762		1.519
—	X	5	2.770**	2.433***	2.644*	2.204***	2.377***	

* Least squares fit failed to converge in single precision and was therefore computed in double precision.

** Calibration curve not acceptable.

same. With the least squares fit for two parameters, the initial guesses of the ordinates are chosen as the logarithm of the high and low molecular weight limits for the packing material given by the manufacturer. For least squares fits with more than two parameters the choice of initial guesses is based on the calibration curve for the previous fit with one parameter less. In the natural cubic spline model the end conditions are chosen so that the second derivative is equal to zero at the end-points, which corresponds to a linear run-out* of the calibration curve in the end intervals. With this choice a somewhat higher X^2 value is obtained compared to those of the other models with the same number of parameters, but it also provides an acceptable calibration curve, where the other models fail, as in the calibration based on M_n with five parameters.

The cubic spline model II represents a compromise between the natural cubic spline model and the polynomial model. For a least squares fit when the number of parameters is less or equal to four, the end conditions are chosen to provide a single polynomial representation. The mathematical equivalence to the polynomials is confirmed by a comparison with the Legendre model. For numerical analysis, however, these models are not identical, since the cubic spline model requires a far smaller number of functional evaluations. When the number of parameters is greater than four, the second derivatives at the end intervals are chosen as parameters, besides the ordinates, which provides more flexibility in the end interval compared to the cubic spline model. However, some of the advantages of the natural cubic spline model will

* If the second derivative approaches zero, the first derivative approaches a constant value.

be lost, and the least squares fits must in certain cases be computed in double precision. Since the spline II model gives a less efficient convergence than the natural cubic spline model, the improvement in the X^2 values can therefore not validate this model, and the natural cubic spline model is then the preferred choice.

The alternative to the cubic spline as empirical model is the polynomial. In least squares fits with polynomials of various degrees, the linear representation was the only one that gave a correct solution for computations done in single precision. Even when these analyses were extended to double precision, some of them failed to minimize X^2 , especially when a large number of parameters were used or only a small number of known molecular weight averages were available. This is a consequence of the strong parameter interaction inherent in the polynomial model. Since the coefficients in the polynomials are weighted by v^i , the values of which are nearly the same over the elution range due to the normalization of the range by the internal standard, the estimate of one parameter is adversely influenced by that of another parameter. Even if this interdependence could be decreased by a suitable coordinate transformation, an orthogonal representation could not be achieved. The ordinary polynomial representation is therefore not a good choice for the numerical analysis.

A better representation for numerical analysis is provided by the Legendre polynomials. With this model an efficient convergence and weak parameter interaction were obtained. However, complete independence of the parameters, which was expected, could not be obtained. In theory, the value of any coefficient is independent of the value of any higher-order coefficients but is not independent of the values of the lower-order coefficients. Each subsequent fit would therefore yield, in addition to the value of the next higher-order coefficient, the same values of the parameters as the previous fit with one parameter less. Each fit would then only be an estimation of a single parameter. Since complete independence of the coefficients could not be realized, some of the fits must be computed in double precision. One possible explanation of this behaviour is that the calibration curve approximation involves an indirect estimation of a function from a set of averages over the elution range, rather than the approximation of a function from a set of data points. However, as the model gives efficient convergence and weak parameter interaction and provides an easy choice of the initial guesses for the parameters, it is a good representation of the calibration curve for numerical analysis.

In the modified polynomial model no fit could be evaluated in single precision due to the strong parameter interaction inherent in the polynomial model. With X^2 values higher than the minimum obtained in the Legendre model, the constant term in this model is not significant. The model must therefore be confined to situations where an adequate number of standards are available, especially in the low-molecular-weight region and to cases where the constant term is significant. Since this model has all the disadvantages of the ordinary polynomial model, a possible improvement would be to combine the first four Legendre polynomials with the constant term. Besides obtaining the advantages of the Legendre model, one could then determine the significance of the constant term by comparing the X^2 value of the Legendre model for four and five parameters with the value obtained for this model.

Of all models, the diffusion model has the unique advantage that the first two parameters can be related to the separation range. Therefore, if the model is to be an acceptable representation of the calibration curve, the estimated values of these param-

eters should coincide with the observed values for the elution range. This is confirmed by the results. Furthermore, with an efficient convergence, low X^2 values and minimal parameter interaction, the model can be considered as a good representation of the calibration curve. However, this is only true if a large number of standards are used for the calibration. The disadvantage of the model is the more complicated numerical treatment due to the inverse relationship of the elution volume and the molecular weight.

The residuals between the experimental and the expected averages are also of value in elucidating the adequacy of the models. In Table III the residuals are given for the three main models. Since the other models are not significantly different from these models they can be excluded from the discussion. With the residuals more or less randomly distributed around zero, the models seems to be an acceptable representation of the calibration curve based on M_n and M_w . More notable perhaps is the small difference between the residuals for the different models. Even if the contribution to the residuals due to the choice of the model is not negligible, this contribution is nevertheless small compared with the total variation. The choice between the three models is therefore not critical, at least not when a larger number of well-characterized standards are available as in this case.

The residuals are also influenced by the limited resolution in the separation process. An estimate of this contribution can be obtained from the quotient between the polydispersity from the GPC measurements and the polydispersity of the standards. If the systematic error due to the limited resolution is not negligible, this quotient will be larger than one. From Table III the mean value of this quotient for the standards with the spline model has been computed to be 1.013. No statistical significance could be attributed to the measured deviation of 1.3% for only seven standards because of the much larger uncertainty in the known molecular weight averages. As the calibration is based on both M_n and M_w , it is possible that the calibration curve model could partly compensate for the increased polydispersity of the GPC method. However, this compensation was not realized for calibrations based on only M_n or M_w . In Table IV the residuals are therefore also given for calibrations based on M_n or M_w for the Spline I model. Since the choice of model is not conclusive, it is probably sufficient to evaluate the effect of the limited resolution for only the natural cubic spline model. For calibrations based on M_n , the mean value of the quotient is 1.010 and for those based on only M_w a value of 1.023 is obtained. The last value is somewhat less reliable since the X^2 value for this fit was 2.195, which is perhaps too high to be acceptable. However, these two values are still approximately the same as for the calibrations based on both M_n and M_w , and it is unlikely that the model has compensated for any excess of polydispersity in the GPC measurements. Thus, within the random uncertainties of the observed and expected molecular weight averages, no significant effect of the limited resolution of the GPC method could be determined.

CONCLUSIONS

For the GPC method based on a least squares approximation of the calibration curve to be valid, no systematic errors should be present. With the low resolution normally obtained for GPC measurements on supports having large particle sizes,

TABLE III

RESIDUAL ANALYSIS OF THE MOLECULAR WEIGHT AVERAGES FOR THREE CALIBRATION CURVE MODELS WITH CALIBRATION BASED ON M_n AND M_w

The models comprised four parameters.

	$1 - M_n/M_n^0$ for standard				$1 - M_w/M_w^0$ for standard									
	T 10	T 20	T 40	T 70	T 110	T 150	T 250	T 10	T 20	T 40	T 70	T 110	T 150	T 250
Spline I	0.006	-0.020	0.065	-0.035	0.018	0.021	0.012	-0.005	-0.008	-0.000	0.013	0.008	-0.066	0.041
Legendre	0.001	-0.014	0.064	-0.044	0.008	0.013	0.009	0.001	-0.009	-0.009	0.016	0.022	-0.057	0.029
Diffusion	0.003	-0.001	0.070	-0.044	0.007	0.015	0.015	0.015	-0.004	-0.009	0.025	0.040	-0.038	0.028

TABLE IV

RESIDUAL ANALYSIS OF THE MOLECULAR WEIGHT AVERAGES FOR THE NATURAL CUBIC SPLINE MODEL WITH CALIBRATION BASED ON M_n OR M_w

The model comprised four parameters.

	Calibration based on M_n				Calibration based on M_w									
	T 10	T 20	T 40	T 70	T 110	T 150	T 250	T 10	T 20	T 40	T 70	T 110	T 150	T 250
$1 - M_n/M_n^0$	0.002	-0.023	0.059	-0.045	0.005	0.008	-0.000	0.064	-0.016	0.066	-0.030	0.024	0.028	0.018
$1 - M_w/M_w^0$	-0.006	-0.015	-0.012	0.002	-0.003	-0.069	0.045	0.002	-0.009	0.004	0.021	0.015	-0.063	0.036

broader GPC distributions would be expected, even if the measurements are performed at low flow-rates. However, within the random uncertainty of the known molecular weight averages, no significant effect of the limited resolution has been observed. In principle, the choice of a mathematical representation of the calibration curve would also be expected to give a systematic error if the model is not an adequate description of the calibration curve. However, for the more generally applicable models such as the spline and the orthogonal polynomial models, where the errors due to the model could be made arbitrarily low by increasing the number of parameters, this error is not realized. The small deviations of the residuals between these models also indicate that the choice of the model is not very important in this case. Nevertheless, in general, the natural cubic spline model is preferred because it can be extended to calibration of multicolumn sets, where a single polynomial may not be appropriate over the calibration range. Since the spline model also provides a linear runout in the end intervals, its use is more acceptable with the normally limited information available for the calibration.

The number of parameters in the model was determined by minimizing the X^2 value. The adequacy of this procedure is seen from the fact that for all fits where the procedure could be applied an acceptable calibration curve was obtained. With the inclusion of the total variances as weighting factors, the possibility of unequal reliability of the known molecular weight averages can be taken into account, resulting in a more correct use of both M_n and M_w for the calibration. Since the total variance in this case is almost completely determined by the uncertainty in the known averages, the contribution of the GPC method to the total error in the calibration could be neglected.

Many of the more common calibration methods used today are based on only a few known molecular weight averages. Even if these methods also correct for the limited resolution in the GPC separation, the dominating effect of the large uncertainty in the known averages is not taken into account. When the GPC calibration is based on a larger number of standards, a more correct non-linear calibration curve is obtained and the effect of the random errors in the known averages is reduced due to the smoothing of the least squares estimation.

Although the calibration procedure in this study has been evaluated only for standards having broad molecular weight distributions, the use of those having narrow ranges is not ruled out. In fact, the only requirement of the procedure is that the known averages can mathematically be related to the molecular weight, in which case the molecular weights of narrow standards *versus* peak elution volume represents the simplest relation.

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