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EXPERIMENTAL EVALUATION OF THE PRECISION IN TIME-BASED HIGH-PERFORMANCE GEL PERMEATION CHROMATOGRAPHY MEASUREMENTS

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SUMMARY

The within-day precision of time-based gel permeation chromatography (GPC) measurements on small-particle supports has been measured as 1.0% for M_n and 1.2% for M_w over the calibration range. This variation is shown to be mainly due to flow-rate variations, which are long term compared to the time scale of the runs. The contribution of detector noise as evaluated by cubic spline data smoothing was found to be negligible. In comparison with the precision in the measurements of the calibration standards by absolute methods such as light scattering and end-group analysis, the random errors in the GPC measurements could be neglected in the calibration procedure. It is thus possible to obtain time-based high-performance GPC measurements on small-particle supports, not only of high accuracy but also of high precision without any correction.

INTRODUCTION

Since its introduction, gel permeation chromatography (GPC) has usually been performed on columns packed with large-particle supports. For the analysis of polymers, GPC measurements have therefore required long columns and long times of analysis to give sufficient resolution. With the development of small-particle supports for GPC, not only an improved accuracy but also a substantial reduction of column length and time of analysis are possible[1].

However, concern has been expressed that flow-rate variations could lead to more significant errors than those in conventional GPC[2]. With the improved accuracy using small-particle supports, the random errors could therefore be the limiting factor in GPC analysis. As the GPC precision will ultimately determine the precision by which a calibration curve can be measured[3], an experimental evaluation has been done to determine the precision in time-based GPC with small-particle supports.

The random uncertainty in the GPC measurements is generally caused by flow-rate variation and noise in the detector signal.

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Flow-rate variation

In GPC on small-particle supports the molecular weight is related to elution time rather than elution volume as a consequence of the small elution volumes. Time-based GPC is therefore dependent on the ability of the solvent delivery system to maintain a constant flow-rate. As the calibration curve often covers a large range of molecular weights over a rather small range of elution volumes, even a small variation in the flow-rate produces large errors in the molecular-weight averages.

The effects of the apparent inability of the present solvent delivery systems to maintain a constant flow-rate can be minimized mainly by two different approaches. In the first method, the average flow-rate during a run is measured by using an internal standard, and the deviation from the flow-rate used at the calibration is compensated for[4,5]. This method can therefore be used only for flow-rate variations from run to run. The second approach offers a more comprehensive solution with a flow feedback system[6]. In this system the actual flow-rate is measured, and the deviation from the desired flow-rate is fed back to the pump to correct the flow-rate. It is thus possible even to correct for flow-rate variations within a run. With these methods it seems possible to obtain a relative standard deviation of 0.1–0.2 % for the retention time. However, since in a recent study of precision in liquid chromatographic measurements[7] a Waters 6000A pump operated under optimal conditions gave a flow-rate with a relative standard deviation of 0.07 % over a period of 12 h without correction, most of the observed flow-rate instabilities can be related to the conditions under which the chromatographic analysis is carried out. The variation due to inherent deficiencies of the solvent delivery system seems therefore to constitute only a minor part of the total flow-rate variation.

Detector noise

In the numerical treatment of GPC data, the noise in the detector signal introduces an uncertainty in the computed molecular weight averages. With a well-designed detector, noise having a frequency significantly higher than that of the peak is normally well filtered. If noise having a frequency in the same range as the signal is to be affected, a more extensive smoothing technique is required.

In this work a cubic spline data smoothing technique[8] has been used to evaluate the detector noise contribution to the total random error. The smoothing has been carried out as follows. A smooth cubic spline, S, is placed along the set of data points, F_i, of the GPC measurements, according to

$$\sum_{i=1}^{n} (S_i - F_i)^2/s^2 \leqslant n$$

where s^2 is the variance of the detector noise and n the number of data points. The equality holds unless S describes a straight line. Of all cubic splines satisfying the equality, the function which minimizes the integral

$$\int_{x_1}^{x_n} S''(x)^2 \, dx$$

is chosen. With this choice a linear runout is obtained corresponding to the baseline. The advantages of this smoothing method are that a global rather than a local smoothing occurs and that the actual amount of noise is taken into account.

EXPERIMENTAL

The degassed solvent, 0.5 m*M* H_2SO_4 solution prepared from Milli-Q water and prefiltered through a 0.2-μm Millipore filter, was gravitationally fed to an Altex Model 110A pump equipped with an optional pressure filter. The flow-rate was 0.2 ml/min in all runs. As preliminary runs gave an unreliable retention-time reproducibility, two modifications of the pump were made. The C13 capacitor on the printed circuit board was removed to eliminate the solvent compressibility compensation and a new ball seat in the inlet check valve was constructed of stainless steel, replacing the original one of sapphire. With these modifications an approximately constant and somewhat better day-to-day flow-rate reproducibility was obtained. The samples, dextran standards (Pharmacia, Uppsala, Sweden) with a concentration of 1.5 μg/μl to which was added ^2H_2O as an internal standard at a concentration of 0.25 μg/μl, were injected with a Rheodyne Model 7010 injection valve with a 10-μl sample loop. For the GPC measurements, two μBondagel E-linear columns (Waters Assoc.) thermostated in a water-bath at 30°C were used. A Multiref 901 (Optilab Instrumentation, Vällingby, Sweden) refractive index detector with a 10-mm measuring cell was chosen because of its high sensitivity. The standard deviation of the baseline noise for a detector time constant of 0.3 sec was measured to be $< 3 \cdot 10^{-9}$ refractive index units; this value was used in the cubic spline data smoothing. A digital panel meter (Analog Devices' AD2008) with an external variable digital time-base provided a dual slope integrating analog–digital conversion with >60 dB of normal mode noise rejection at line frequency. The room containing the total GPC system was thermostated at 25°C.

For the replicate GPC determinations, a series of seven broad dextran standards was run in random order on three separate days with three series each day. The cubic spline model[3] was used to represent the calibration curve. Unbiased estimates of the molecular weight averages were obtained by determining the baseline of the GPC curve by computer.

RESULTS AND DISCUSSION

The flow-rate variations as measured by use of the internal standard (IS) are plotted in Fig. 1. As indicated by the deviations from the mean value for each day, no significant daily drift was observed. A shift of the mean value was observed for the third day, but runs over a period of a few months did not show an accumulative drift in the elution counts of the IS. Anyhow, such a shift in the elution counts from day to day could be compensated for with the variable digital time-base.

From the mean value of the variances for the three days, the within-day precision was estimated as 0.07%. This value corresponds to a standard deviation of *ca.* 4 μl, which should be compared with the piston displacement volume of 140 μl. If the flow noise due to insufficient pulse damping is the major cause of the observed flow-rate variation, the deviations should be more or less randomly distributed. Fig. 1

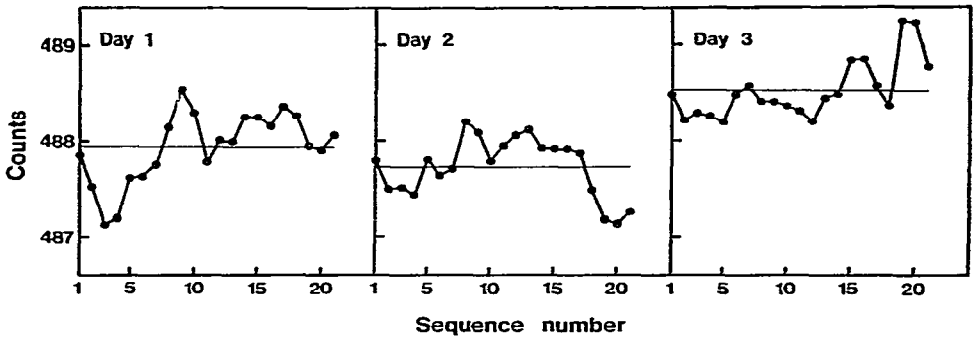

Fig. 1. Retention counts of the internal standard (1 count = 3.50 sec).

implies that the main flow-rate variations are long term compared to the time scale of the individual runs.

With the low sample load (15 μg) used for the runs, no influence of the molecular weight of the dextran standards on the flow-rate was expected. This has been verified by the mean elution counts of the IS computed for each dextran standard each day (Table I). Since the variation in the mean retention count is randomly distributed with the molecular weight of the dextran standards, there is no correlation between the molecular weight and the flow-rate.

TABLE I

MEAN RETENTION TIME OF THE INTERNAL STANDARD FOR EACH DEXTRAN FRACTION

Retention time in counts with one count equal to 3.50 sec.

	*Standard**						
	T 10	*T 20*	*T 40*	*T 70*	*T 110*	*T 150*	*T 250*
Day 1	487.6	488.2	487.9	488.0	487.8	487.8	488.0
Day 2	487.5	488.1	487.6	487.6	487.7	487.6	487.7
Day 3	488.5	488.5	488.6	488.8	488.5	488.6	488.6

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

The observed variation in the elution time of the IS could be caused by an uncertainty in the time of injection. The standard deviation of 4 μl, corresponding to 1.2 sec in the elution time with the flow-rate used, is too large, however, to be explained as an injection error. The measured standard deviation of 0.2 sec for runs without columns confirms this.

The relative standard deviations of the M_n and M_w values for each dextran standard measured within the same day are given in Table II. In the steep parts of the calibration curve, where a small change in the elution volume leads to a large change in the molecular weight, a greater relative standard deviation is expected. This has been observed in the larger $s(M_n)$ value of T 10 and $s(M_w)$ values of T 150 and T 250.

TABLE II

RELATIVE STANDARD DEVIATIONS (%) FOR MOLECULAR WEIGHT AVERAGES OBTAINED FOR DEXTRAN STANDARDS BY TIME-BASED HIGH-PERFORMANCE GPC

	Standard						
	T 10	*T 20*	*T 40*	*T 70*	*T 110*	*T 150*	*T 250*
Day 1							
$s(M_n)$	1.93	0.71	0.51	0.81	0.23	1.33	1.48
$s(M_w)$	1.41	0.59	0.58	1.02	0.68	1.60	2.28
Day 2							
$s(M_n)$	0.95	0.47	0.73	1.09	0.75	1.16	0.37
$s(M_w)$	0.62	0.40	0.69	1.17	1.09	1.57	0.66
Day 3							
$s(M_n)$	0.97	0.25	0.81	0.63	0.52	1.95	0.62
$s(M_w)$	0.54	0.06	0.68	0.98	0.75	2.76	1.30

There exists no correlation between the molecular weight and the precision for the linear part of the calibration curve. Since the precision is essentially independent of the molecular weight, the within-day precision of time-based GPC has been estimated to be 0.99% for M_n and 1.20% for M_w from the mean value of the variances for all standards.

The flow instabilities consisting mainly of long-term variations could be compensated for by use of the IS. From the results given in Table III, values for the within-day precision of 0.42% for M_n and 0.58% for M_w are obtained for flow-corrected GPC. Comparison with time-based GPC confirms that the flow noise can be neglected as a source of error.

TABLE III

RELATIVE STANDARD DEVIATIONS (%) FOR MOLECULAR WEIGHT AVERAGES OBTAINED FOR DEXTRAN STANDARDS BY TIME-BASED HIGH-PERFORMANCE GPC WITH FLOW-RATE VARIATION CORRECTED BY USE OF INTERNAL STANDARD

	Standard						
	T 10	*T 20*	*T 40*	*T 70*	*T 110*	*T 150*	*T 250*
Day 1							
$s(M_n)$	0.46	0.38	0.15	0.57	0.49	1.00	0.64
$s(M_w)$	0.20	0.38	0.19	0.65	0.76	1.21	1.15
Day 2							
$s(M_n)$	0.15	0.21	0.16	0.68	0.33	0.31	0.17
$s(M_w)$	0.19	0.24	0.18	0.57	0.68	0.59	0.07
Day 3							
$s(M_n)$	0.13	0.15	0.24	0.23	0.25	0.57	0.16
$s(M_w)$	0.02	0.14	0.23	0.50	0.38	1.00	0.60

The results from the flow-corrected GPC also indicate that the contribution of the detector noise to the total error must be small. In Table IV, the results of time-based GPC measurements smoothed with the cubic spline technique are given for a comparison with time-based GPC. Since the precision, 0.95% for M_n and 1.19% for M_w, is approximately the same as without signal-to-noise enhancement, smoothing of the data is unnecessary.

TABLE IV

RELATIVE STANDARD DEVIATIONS (%) FOR MOLECULAR WEIGHT AVERAGES OBTAINED FOR DEXTRAN BY TIME-BASED HIGH-PERFORMANCE GPC WITH CUBIC SPLINE SMOOTHING OF DATA

	Standard						
	T 10	*T 20*	*T 40*	*T 70*	*T 110*	*T 150*	*T 250*
Day 1							
$s(M_n)$	0.59	0.69	0.60	0.79	0.23	1.29	1.83
$s(M_w)$	1.15	0.56	0.61	1.09	0.67	1.65	2.40
Day 2							
$s(M_n)$	1.04	0.33	0.67	1.07	0.79	1.10	0.37
$s(M_w)$	0.68	0.35	0.76	1.21	1.12	1.51	0.58
Day 3							
$s(M_n)$	1.13	0.15	0.78	0.72	0.54	1.97	0.90
$s(M_w)$	0.56	0.07	0.67	0.99	0.70	2.62	1.41

The refractive index detector normally used for GPC measurements has by design a moderate sensitivity. With the Optilab 901 refractive index detector, which is based on an interferometric method, higher sensitivities could be used at low time constants. An acceptable signal-to-noise ratio could therefore be obtained even when the sample load had been made sufficiently low to minimize any influence on the GPC performance.

The precision of time-based GPC must also be compared with the precision of absolute methods such as end-group analysis and light scattering, since the latter methods established the M_n and M_w values for the calibration standards on which the calibration is based[3]. With a relative standard deviation of 5% for M_n and 3% for M_w (estimated from data in ref. 9), the variances of the GPC measurements could be neglected in comparison with the variances of the absolute methods. The precision of the calibration is thus limited by the precision of the absolute methods and not by the precision of GPC, and runs of a single set of the calibration standards are sufficient to define the calibration curve.

With the development of small-particle supports for GPC, an accuracy of 2% in the molecular weight averages is possible[1]. Even if the precision of time-based GPC cannot be maintained as low as 1% during runs over longer periods, the error due to random variations is still of the same magnitude as the error due to the limited resolution of the GPC method.

In conclusion, for the GPC system used in this work the experimental evaluation of random errors has shown that the small observed errors can be related to long-term flow-rate variations with respect to the time of analysis. The flow and detector noise, which could be expected to contribute due to the small elution volumes and low sample load, are of minor importance. If the low-cost motor-driven single-piston pump used in this work can provide a reproducible and uniform flow under optimal conditions, it is expected that the more advanced pump design normally used for GPC analysis should exhibit at least equivalent performance under similar conditions. Time-based GPC on small-particle supports has therefore the capability of providing such a high precision that the contribution of the random errors to the total errors can be neglected.

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