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NIST-Sponsored Interlaboratory Comparison of Polystyrene Molecular Mass Distribution Obtained by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry: Statistical Analysis

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The method of preparation and methods of analysis of a narrow distribution polystyrene of ∼7 ku used in an interlaboratory comparison of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of synthetic polymers is described. Size exclusion chromatography was used to measure the polystyrene sample variability. Fourier transform infrared spectroscopy and MALDI-TOF-MS were used to analyze end groups on the polymer. The polystyrene was analyzed by MALDI-TOF-MS and classical methods of polymer characterization. The number (M_n) and mass (M_w) average of the molecular mass distribution (MMD) determined by the classical methods (light scattering and NMR) were compared with those obtained by MALDI-TOF-MS. Agreement between classical methods to obtain the moments of the MMD and the MALDI is found to be good overall. However, all the experimental values obtained by MALDI fell below the classical values. A discussion of why these values are lower is included. We discuss the statistical analysis of the data from the interlaboratory comparison conducted by NIST, which includes data from 23 different laboratories. Analysis of variance is used to examine the influences of the independent parameters (laboratory, matrix, instrument manufacturer, instrument mode) on the data. The parameters, laboratory and instrument manufacturer, were determined to have an influence on the MMD, where matrix and instrument mode were found not to have a significant influence.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)[1−4] is a new and important technique in characterization of synthetic polymer molecular mass distribution (MMD).[5−12] Yet much is still unknown about the repeatability and accuracy of the molecular mass distribution as measured by the MALDI-TOF-MS instruments. As the number of polymer analyses by MALDI has increased, scrutiny of the MALDI results in comparison to classically obtained values for M_w and M_n, defined as the mass-average molecular mass and the number-average molecular mass, respectively, has resulted.[13] In some instances, it is found that the results of MALDI and classical analysis do not always agree.[4,5,7,14] One method to obtain a measure of the robustness of a measurement is to compare results of that measurement on the same material between a number of laboratories. To this end, the National Institute of Standards and Technology (NIST) Polymers Division has initiated an interlaboratory comparison to compile MALDI-TOF-MS data, to learn more about the MMD obtained, to identify the parameters that influence the measured distribution, and to compare the results from MALDI with those obtained from classically measured values of M_w and M_n. This paper describes the MALDI-TOF-MS analysis results of 23 respondents (laboratories) that participated in the interlaboratory comparison, with all laboratories analyzing identical polystyrene (PS) samples of nominal molecular mass 7000 u. This polystyrene will ultimately be available as a NIST standard reference material (SRM 2888). (Participating laboratories are listed in Appendix A.) The M_n and M_w of the polystyrene were determined by classical methods at NIST. End groups were further

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studied by Fourier transform infrared spectroscopy (FT-IR) and MALDI-TOF-MS at NIST.

The outline of this paper is as follows. The synthesis and homogeneity testing for the polymer is described in section 1. In section 2, we describe the experimental work done at NIST to measure the moments of the MMD and the identification of the end groups. A brief description of our protocol for the interlaboratory comparison is then given in section 3. In section 4, two kinds of descriptors of the data are defined, including traditional polymer moments of M_w and M_n. The statistical analysis of all the data using these descriptors is given in section 5. The effects of various parameters are described in section 6, including the distinction between and within laboratory and the effects of choice of matrix materials. Finally, in section 7, we try to draw some conclusions from our findings.

1. PREPARATION OF MATERIALS AND HOMOGENEITY TESTING

Synthesis. The PS used in this interlaboratory comparison was prepared commercially by Polymer Source (Dorval, Québec, Canada).[15] The polymer was specially prepared by anionic polymerization with well-defined styrene and tertiary butyl end groups. From the preparation chemistry, we expected the polymer to be atactic polystyrene of the form

$$(CH_3)_3-C-[CH_2-CHPh]_n-CH_2-CH_2-Ph \quad Ph = \text{phenyl} \tag{1}$$

Homogeneity and Bottling. For the interlaboratory comparison, 30 sample vials of ~0.4 g/vial were prepared. Homogeneity testing was done on the vials by size exclusion chromatography (SEC) with samples selected by stratified random sampling.[13] A Waters 150-C ALC/GPC liquid chromatograph (Waters Corp., Milford, MA)[15] with a differential refractive index (DRI) detector was used in this study. Tetrahydrofuran (Mallinckrodt Specialty Chemicals, Paris, KY)[15] with added antioxidant, 2,6-di-*tert*-butyl-4-methylphenol (commonly known as butylated hydroxytoluene or BHT), was used as the solvent. Toluene at 0.3 g/L was added to the solvent used in preparing solutions as an SEC pump marker.

Two solutions in tetrahydrofuran were made from each polymer sample vial. The polystyrene samples were dissolved in the solvent at a concentration of ~1.0 g/L. The order of preparing the solutions and running the chromatograms was randomized. SEC was performed on these solutions using two injections from each solution.

After baseline subtraction, the SEC chromatograms were normalized to unit peak height and compared initially by overlaying to decide whether there were visible differences outside the noise. The chromatograms from different solutions all superimpose on each other. This preliminary comparison showed that polymer samples taken from all the vials produced identical chromatograms. A statistical analysis using methods of comparing chromatograms used in this laboratory for looking at bottle-to-bottle variation of NIST standard reference materials (SRMs) confirmed these observations.[16] Details of that work are to be published as a NISTIR.[17]

2. POLYMER CHARACTERIZATION

Light Scattering Methods To Determine M_w. Light scattering measurements on toluene solutions of the PS were made on a Brookhaven Instrument model BI-200 (Brookhaven Instrument Corp., Ronkonkoma, NY)[15] light scattering apparatus with a 10-mW He−Ne laser light source. In all experiments, the intensity measuring system was calibrated with the intensity of light scattered from a benzene standard cell. The scattering intensity from each polymer solution sample was measured at nine angles in the range from 37.5° to 142.5°. Light scattering data from polymer solutions of concentration c and scattering angle x were fit following normal Zimm analysis.[16] The constant multiplying M_w depends quadratically on (dn/dc), the change in refractive index of solution as a function of concentration. Normally, for homopolymers, dn/dc is independent of molecular mass. At lower molecular masses, however, because the refractive increments from the end groups are expected to differ from that of the repeat units, we usually find the dn/dc to have the form

$$dn/dc = A + B/M_n \tag{2}$$

For the PS polymer used in the intercomparison, we measured dn/dc of the polymer in toluene and found the dn/dc to be 0.1030 ± 0.0010 mL/g, where 0.0010 mL/g is the standard deviation. With this dn/dc, we estimate $M_w = 7.19 \pm 0.56$ ku. The expanded uncertainty of 0.56 ku includes both repeatability, which is estimated by a type A evaluation of uncertainty, and systematic uncertainty, a type B evaluation of uncertainty.[18] Methods of data and uncertainty analysis used to estimate the M_w obtained here are described in ref 16. A complete description of the analysis for this polymer will be published with the SRM report.[17]

NMR Methods to Determine M_n. Proton NMR spectra at 400 MHz were run at ambient temperature on a WM-400 spectrometer manufactured by Bruker Instruments, Inc. (Billerica, MA).[15] Resolution was found to be adequate for evaluating the integrals of interest under the following conditions. Solutions were prepared of 5% and 13% PS in deuterated benzene (benzene-d_6). Spectra were taken with single pulse excitation. Pulse nutation ("tip'") angle was 30° and the delay between acquisitions was 20 s. This combination of conditions was confirmed to give quantitative results for all protons. Signal accumulations after 64 scans had adequate signal-to-noise ratios in the Fourier transform spectra for evaluating the integrals of interest. The Fourier transforms were made large enough, by zero-filling, so that the relative integrals, for even the narrowest lines, were reliable. Integrals on the NMR spectra of the PS were measured. On the basis of the assumed structure for the PS shown in eq 1, the integrals (of both aromatic and aliphatic PS protons plus the end

(15) Certain commercial equipment is identified in this article in order to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the items identified are necessarily the best available for the purpose.

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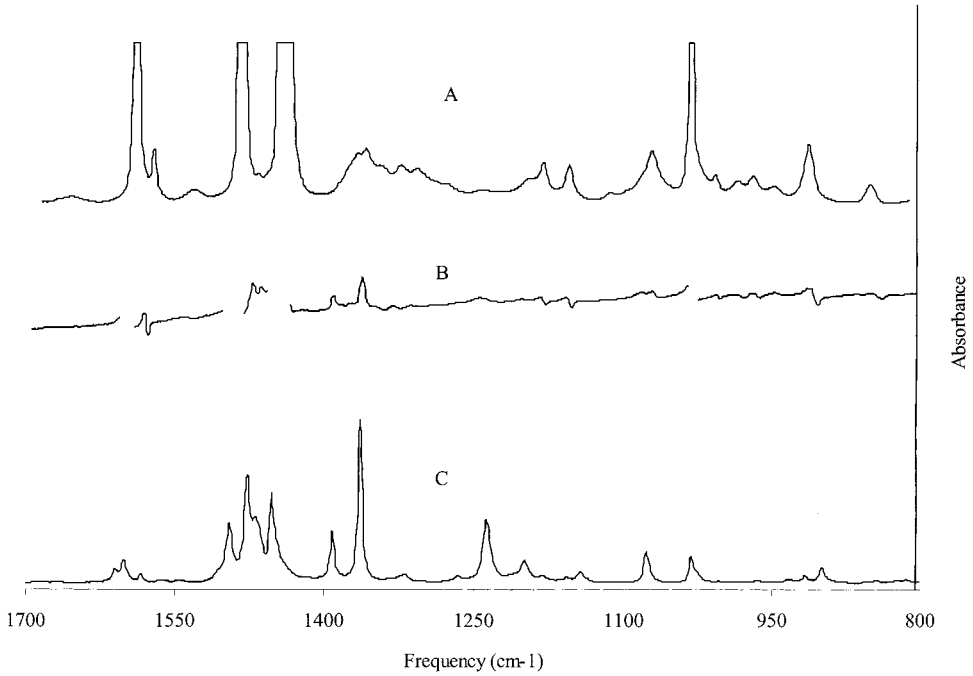

Figure 1. FT-IR spectrum of the MALDI PS (A), neopentylbenzene (C), and the difference spectrum, MALDI PS − SRM1479 (B). Peaks identifying the *tert*-butyl end groups are at 1365, 1393, and 1475 cm^{-1}.

group methyl protons) measured from the NMR, yield an M_n of 7100 u based on measurements from the 5% solution and an M_n of 7000 u based on the measurements of the 13% solution. The 13% solution had worse resolution, as expected from a more concentrated solution, but smaller corrections had to be made to the measured integrals than for the 5% solution. Thus, the confidence level for each solution was similar. We estimate from NMR the $M_n = 7.05 \pm 0.40$ ku. The expanded uncertainty of 0.40 ku includes both repeatability, determined by a type A evaluation of uncertainty, and systematic uncertainties, evaluated as a type B uncertainty.[18] A complete description of the analysis for this polymer will be published with the SRM report.[17]

FT-IR Determination of the *tert*-Butyl End Groups. Infrared spectroscopic analysis was used to confirm the identity of the end groups and to identify the existence of minor chemical impurities that may be present at detectible levels in the as-received material. According to the synthesis, each polystyrene molecule should contain a *tert*-butyl group at one end and a hydrogen atom at the other end (as seen in eq 1). Whereas the latter group is difficult to discern in the infrared spectrum, the *tert*-butyl group is easily identified at the 1:70 molar ratio with styrene repeat unit expected with this polystyrene. The FT-IR spectrum of the MALDI PS, average of 200 scans at 1.0-cm^{-1} resolution, is shown as the top trace in Figure 1.

As a model infrared spectrum of polystyrene terminated by a *tert*-butyl group, the infrared spectrum of neopentylbenzene, 2,2′-dimethylpropylbenzene, was recorded and shown as the bottom trace in Figure 1. Two bands, at 1365 and 1393 cm^{-1}, are present in this spectrum that are identified with motions of the methyl groups of the *tert*-butyl group.[19] Another intense band, also attributable to the *tert*-butyl group, occurs at 1475 cm^{-1}. All three of these bands are evident in the infrared spectrum of the MALDI PS, top trace in Figure 1. To enhance visualization of contributions to the infrared spectrum from end groups of PS, or chemical impurities to the extent that they exist at concentrations comparable to end groups, the spectrum of high molecular mass polystyrene, SRM 1479 (M_w = 1 050 000 u), was recorded and subtracted from the spectrum of the sample polystyrene to remove the "normal" polystyrene contributions. The resultant difference spectrum appears as the middle trace in Figure 1. The infrared difference spectrum between the MALDI PS and SRM 1479 contains three bands at 1365, 1393, and 1475 cm^{-1} that are characteristic of the *tert*-butyl group. Although the difference spectrum contains several other bands of comparable magnitude, these appear at frequencies identical to normal infrared bands of polystyrene, and for this reason, these bands cannot be unambiguously assigned to end groups or impurities. The absence of other bands in the difference spectrum at comparable or greater intensities suggests no chemical impurities are present with concentrations greater than 1%.

Preliminary MALDI at NIST. A preliminary MALDI analysis on the PS was done at NIST on a Bruker Reflex II MALDI-TOF-MS (Billerica, MA) to see if the PS fulfilled the requirements of the interlaboratory comparison.[15] The polystyrene sample was expected from the preparation chemistry to consist of oligomers of the form shown in eq 1. The spectral main peaks from a calibrated instrument agreed well with the structure in eq 1; see Figure 2. However, MALDI mass spectrum of the sample revealed an unexpected secondary series of peaks, also with 104 u mass separations, in addition to the expected main series ions; see Figure 3. We were concerned that some of these intermediate peaks indicated end groups not seen in the FT-IR. Additional experimentation on the polystyrene sample revealed that the secondary series peak position changed with respect to the main series peaks when different matrixes were used. Postsource decay[20] was used to determine that the secondary peaks arose from two sources: either adducts of the matrix and/or cations

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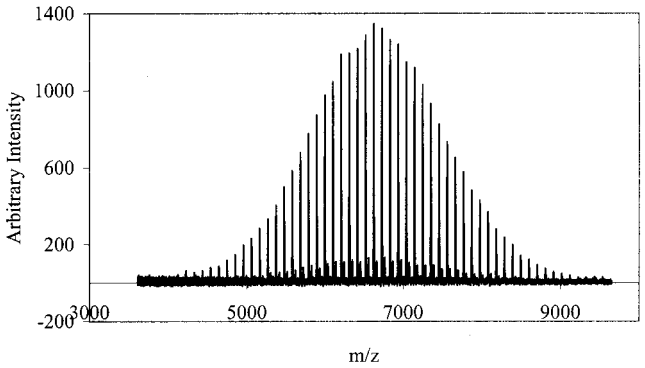

Figure 2. MALDI-TOF-MS MMD of Interlaboratory Comparison Polystyrene using the specified recipe of retinoic acid and AgTFA.

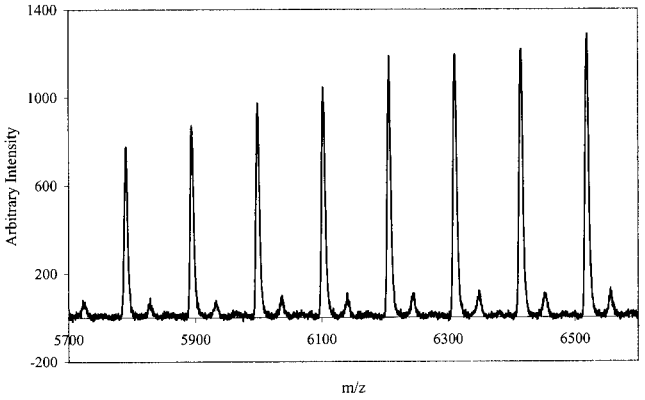

Figure 3. MALDI-TOF-MS MMD of PS expanded to show the secondary peak series.

with the polymer or fragmentation of the polymer along the main chain. The matrix salt adducts cause the secondary peaks to shift when different matrixes are used. None of the secondary peaks were attributable to additional end groups. Details of how these attributions were established are given in ref 20.

3. INTERLABORATORY PROTOCOL

The protocols for the interlaboratory comparison were decided on by a steering committee of MALDI-TOF-MS users organized from membership of the Polymeric Materials Interest Group of the American Society for Mass Spectrometry. Each participating laboratory was asked to perform MALDI mass spectrometry using two protocols. The different protocols involved different sample preparations. The first of the protocols was specified. This protocol requires retinoic acid for the matrix and AgTFA for the salt.[10] The specified protocol is listed in Appendix B. The second protocol allowed each laboratory to use a sample preparation of their own choosing. Each laboratory was asked to produce three MALDI spectra for each protocol to check for intralaboratory variability. Six spectra are obtained from two sample preparations for each laboratory. Each laboratory was asked to provide M_n and M_w for each repeat as well as the integrated mass intensity signal for each separate peak of the PS mass spectrum with the cation mass subtracted from the peak masses. The M_n and M_w values used in the following discussions were obtained from the analysis of integrated signal peak intensities reported by the participants, rather than their values of M_n and M_w.

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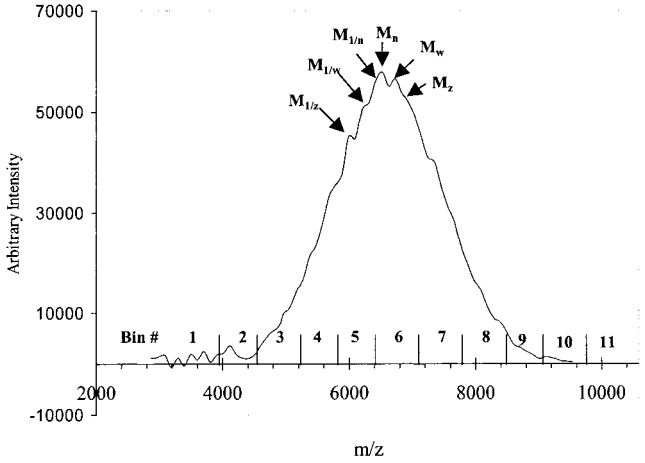

Figure 4. Moments and bins shown in relation to the integrated PS MMD. The moments represent the center of the MMD, whereas the bins represent the entire distribution.

4. ESTIMATORS OF THE MOLECULAR MASS DISTRIBUTION (MMD)

The integrated peak intensities received from the participants were first reduced into estimators, which were then compared and interpreted using statistical methods. The data were reduced into two types of estimators.

The first data reduction method was to use the moments of the molecular mass distribution. Six moments of the MMD were considered. M_n, M_w, and M_z are the traditional moments used in polymer molecular mass determination[13] and have been defined many times before (for examples, see ref 13). We propose here to use three other moments that we call, $M_{1/n}$, $M_{1/w}$, and $M_{1/z}$. These moments are defined as

$$M_{1/n} = \frac{\sum N_i}{\sum N_i M_i^{-1}} \qquad M_{1/w} = \frac{\sum N_i M_i^{-1}}{\sum N_i M_i^{-2}} \qquad M_{1/z} = \frac{\sum N_i M_i^{-2}}{\sum N_i M_i^{-3}}$$

where N_i is the number of moles of molecules with a molecular mass of M_i.

These newly formed reciprocal moments weigh the smaller masses of the distribution heavier than the larger masses of the MMD. These six moments were then compared using the statistical techniques to be discussed later.

These six moments do not adequately represent the tails of the narrow molecular mass distribution for the PS. Even though the newly defined moments are defined to more heavily weight the lower molecular masses in the distribution, for such a narrow MMD the center of the distribution still dominates these moments. (See Figure 4.) The high- and low-mass tails of the molecular mass distribution are expected to have the most variation among laboratories owing to lower peak intensities. Thus, it is important to consider their influence in the data analysis. A second method of reducing the data for analysis was considered. The molecular mass distribution of each data set was separated into 11 mass divisions, bins, before comparison. The bins are taken to be six PS repeat units, 625 u, in width, except for bins 1 and

Table 1. Mean, Standard Deviation, and Standard Uncertainty of the Moments Obtained by All the Laboratories and Both Protocols

	$M_{1/z}$	$M_{1/w}$	$M_{1/n}$	M_n	M_w	M_z
total mean	6131.6	6291.8	6443.9	6587.8	6724.3	6853.9
std deviation	218.8	173.8	137.3	111.1	96.5	93.0
std uncertainty	26.5	21.0	16.6	13.4	11.7	11.2

11, which contain the remaining area of the tails. The bin area of the distribution is then used for the statistical comparison. This method is particularly beneficial to compare the effects of the tail regions of the molecular mass distribution.

5. DESCRIPTION OF OVERALL DATA

Mean Moments and Histogram of MMD. The mean number-average molecular mass (M_n) of the entire data set, using all instruments and both protocols, was found to be 6609.89 u. The standard deviation (σ) was found to be 120.64 u, and the standard uncertainty of the mean ($\sigma/\sqrt{N}$) was 11.77 u,[21] where N is 105. The standard deviation is approximately equivalent to one repeat unit of polystyrene, 104 u, giving a very narrow distribution of data. The mean moments, the standard deviations, and the standard uncertainty of the mean are given in Table 1. The uncertainty is greater for the reciprocal moments, which represent the smaller masses of the molecular mass distribution. This suggests that the low-mass tail of the distribution, which is better represented by the reciprocal moments, has a greater uncertainty than the center of the mass distribution.

Table 2 shows the total means of the bin data. It also shows the standard deviation and the standard uncertainty of the mean for the bins of the MMD. The bins are normalized, therefore indicating the fraction of the total MMD that they contain. From the bin means, we see that bins 1 and 2 make up less than 4% of the MMD. Also bins 9−11 make up less than 4% of the MMD as well.

These data are also represented in Figure 7, which illustrates the mean distribution of the bins for the data obtained using protocol 1. The polystyrene analyzed was made by anionic polymerization. When anionic polymerization is used to make a polymer, a very narrowly distributed polymer is produced. The histogram of the mean bins in Figure 7 is Gaussian.

Outliers. The outliers were determined and removed from the data to prevent erroneous influences on the data analysis. Only the number-average molecular mass (M_n) was considered in the identification of outliers. Since the M_n data represent a distribution of means, the assumption can be made that the M_n moments are normally distributed. A normal probability plot was examined to ensure the assumption of normally distributed data.[22] Also, an F test comparing the shape of the sample distribution to the shape of the normal distribution was found to be have a p-value of 0.3995 (where $0 \leq p \leq 1$). The p-value is the area in the upper tail of the corresponding F distribution, and a p-value less than the significance level of the test (α) provides evidence against the sample distribution being considered normal.[22] A p-value of 0.3995, when $\alpha = 0.05$, fails to reject the hypothesis that the sample distribution is normally distributed.

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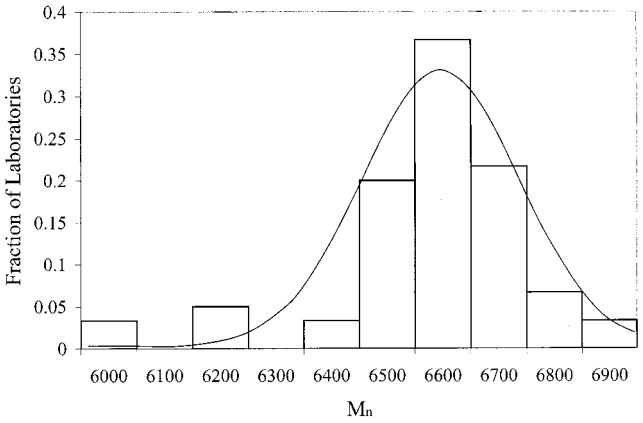

Figure 5. Histogram representing the distribution of M_n. The graphed line represents the normal distribution for the data, and the outliers are seen at 6200 and 6000 u.

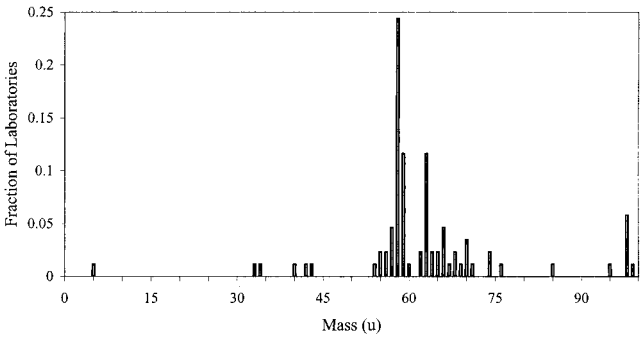

Figure 6. Distribution of the end group masses, which were calculated from the maximum peak values. The end groups, which are hydrogen and *tert*-butyl, should have a mass of 58 u.

Since the M_n distribution is normally distributed, a normal distribution can be used to identify the outliers of the distribution. Three standard deviations of a normal distribution contain 99.8% of the data, so any values that lie outside of this range are considered to be outliers.[19] Figure 5 shows the distribution of the M_n data and the fitted normal curve for these data. Three laboratories obtained mass distributions, which yielded moments that fell outside of three standard deviations of the mean of the normal distribution. For the purposes of this analysis, these data points were classified as outliers and excluded from further data analyses.

Further inspection of the molecular mass distributions from these data sets suggested that for two of the laboratories (respondents) the moments fell outside of the normal distribution, not due to an uncertainty in the data collection and measurement by the MALDI-TOF-MS but due to misinterpretation of the data analysis for the obtained MMD. Of the laboratories that were determined to be outliers, one either seemed not to have performed a baseline correction or improperly corrected the baseline. Another of the laboratories integrated incorrectly, thereby eliminating the high-mass tail data. We saw effects of these data analysis problems in reports from nonoutlying laboratories as well, although the problems were less severe, allowing the moments to fall within the accepted data range. We therefore conclude that the need for significant improvement in data analysis methodology is the first important finding from this interlaboratory comparison.

Low and High Molecular Masses in the MMD. Due to the method of assigning bins, some laboratories' results contained

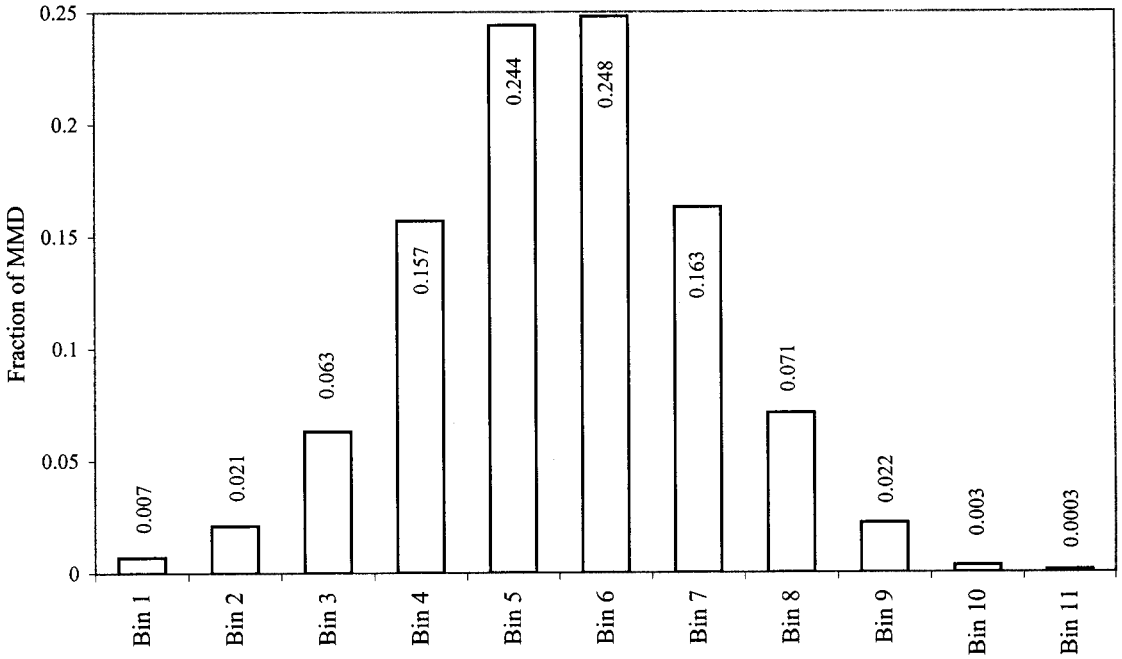

Figure 7. Histogram of the distribution of the mean bins. The fraction of the MMD is given on the histogram as well.

Table 2. Mean, Standard Deviation, and Standard Uncertainty of the Mean for the Bins of the MMD

	bin 1	bin 2	bin3	bin 4	bin 5	bin 6	bin 7	bin 8	bin 9	bin 10	bin 11
total mean	0.0074	0.0204	0.0633	0.1575	0.2437	0.2482	0.1634	0.0709	0.0219	0.00303	0.000296
std deviation	0.0125	0.0152	0.0159	0.0301	0.0174	0.0224	0.0202	0.0155	0.0105	0.00417	0.000935
std uncertainty	0.00154	0.00187	0.00196	0.00370	0.00214	0.00275	0.00248	0.00191	0.00129	0.000514	0.000115

no data in the bins representing the tail region of the MMD. For the low-mass tail of the distribution, 55% of the laboratories reported no data in bin 1 and 10% reported no data in bin 2. The missing data were more extreme in the high-mass tail, presumably due to a loss of instrument sensitivity in the high-mass region. In bin 10, 45% of the laboratories' resulting data sets contained no data, and 85% of data sets contained no data in bin 11. The loss of data in the tail regions may be a result of instrument sensitivity and resolution or may be a result of baseline correction and integration. Each of the different instrument types produced data sets that lacked data in the tails of the distribution. From some limited experience with looking at the above-mentioned outlier data, we would suggest some missing data are attributable to the influence of the integration methodology. Depending on the software used, peaks in the tail regions with baseline noise are very easily missed by peak selection software.

Instrument Calibration. The accuracy of the instrument calibration of each laboratory was assessed by calculation of the end group mass. The masses of the end groups of the polystyrene were calculated by taking the difference between the mass of the maximum signal of the distribution and the calculated mass from the number of repeat units; the cation mass has already been subtracted. Figure 6 shows the distribution of the calculated end group masses. The end groups are *tert*-butyl and hydrogen (as seen in eq 1) and have a total mass of 58.14 u. We would expect the calibration of most TOF mass spectrometers to be accurate to less than 3 u, but as can be seen in Figure 6, some laboratories were over 40 u off.

We considered whether the inaccuracy of the instrument calibrations would cause uncertainties in our analysis. When compensations were made to M_n due to these calibration discrepancies, the value of the mean M_n was only slightly altered, and the variance of the M_n values decreased slightly. When the corrected M_n was analyzed, the results of the analysis in the following sections were not altered. Therefore, the corrections were not continued in the statistical analysis described below.

6. EFFECT OF PARAMETERS ON THE MMD

In the analysis of the interlaboratory comparison data, several parameters were considered as possible influences on the polystyrene molecular mass distribution. The parameters examined were effect of laboratory, effect of sample preparation, effect of instrument manufacturer, and effect of TOF-MS mode (reflectron or linear). Whether the laboratory in which the polymer is examined has an influence on the MMD is an important test of the robustness of the MALDI-TOF-MS method of polymer characterization. The type of matrix used in sample preparation of the polymer for MALDI analysis is also a very significant parameter. The two matrix preparations that were compared in this analysis were *all-trans*-retinoic acid and dithranol. Other matrix preparations were used, but not by enough laboratories, so we were unable to include them in the comparison. The instrument parameter tests differences in the types of instruments, which were Bruker, PerSeptive, Micromass, Physical Electronics' Trift, ThermoBioanalysis Vision, and homemade instruments. The parameter instrument classifies the laboratories by instrument

manufacture, not the model of the instrument. The mode of the instrument is tested to determine an influence on the MMD when the TOF-MS is run in linear or reflectron mode.

Statistical Methods To Describe the Data. Analysis of variance (ANOVA) is a standard statistical analysis tool, which uses sample data to make inferences about populations.[23] The ANOVA test indicates differences in population means by comparing the variation between the treatments (experimental conditions) with the variation within treatments. If the between treatment variation differs greatly from the within treatment variation, the means of different treatments are concluded not to be equal. If the between and within variations are approximately the same size, then there will be no significant difference between treatment means.

Two-way ANOVA assesses the effects of two parameters on the response variable. The analysis considers that effects due to one parameter may mask the effects due to the second parameter. The effects of each factor are called main effects and one, both, or neither may turn out to be significant. In addition to these main effects (and independent of them), there may be an effect due to their interaction. The interaction effect accounts for how simultaneous changes in the two parameters affect the response variable.

Effect of Laboratory on the MMD. The statistical analysis of the parameter laboratory only included data that were taken using the prescribed recipe, protocol 1, for sample preparation. One sample was prepared, and three spectra were taken for that sample. Therefore, if an effect of laboratory exists, it may be due also to a sample preparation effect. After outliers were identified and removed, as well as laboratories that did not include three mass spectra repeats of the polystyrene, 16 laboratories were included in the analysis.

First a one-way ANOVA of the moments for the parameter, laboratory, was performed on the data. The results showed that the parameter laboratory has a significant effect on the molecular mass distribution. The ANOVA of the bins for the laboratory parameter revealed that laboratory, in which the MALDI analysis of the PS is performed, has a significant effect on each of the bins of the molecular mass distribution. Surprisingly, all of the bins, even the center bins, which are expected not to be as sensitive to the parameters as the bins representing the tails, showed a significant variation among laboratories.

But the one-way ANOVA of the laboratory parameter does not give conclusive results, because the instrument parameter and laboratory parameter are confounded. Two parameters are confounded if their effects on the response variable, in this case the MMD, cannot be distinguished from one another. The confounding exists because each laboratory has only one instrument type. Therefore, other methods of analysis are needed to differentiate the two effects.

A method of statistical analysis that can be used to analyze the effect of laboratory, which accounts for the confounding of the instrument parameter, is a two-way ANOVA. The two-way ANOVA first accounts for the effect of instrument. The effect of laboratory is then considered. If the laboratory parameter explains additional effects, then the laboratory parameter is significant. Because of the confounding of the instrument and laboratory parameters, the data were reduced further to include only those instruments run by multiple laboratories, leaving 13 laboratories in the statistical analysis. In the two-way ANOVA, when the instrument parameter is accounted for, the laboratory parameter is found to have a significant effect on all of the moments and all of the bins, representing the molecular mass distribution of polystyrene.

Effect of Instrument on the MMD. The instrument variable considers all instruments from the same manufacturer together as one parameter, regardless of the model of the instrument. There were six different instrument types identified in our study. These were Bruker, PerSeptive, Physical Electronics' Trift, Micromass, ThermoBioanalysis Vision, and several homemade instruments. Of these only three instrument types were used by more than one laboratory, so only the 13 laboratories that ran one of these three instrument types were included in the analysis. As well, only the defined protocol data were considered in the statistical analysis.

To determine the effect of instrument on the molecular mass distribution of the polystyrene, the laboratory parameter must be removed from the data. This was achieved by taking the mean of the three moments (or bins) of the three repetitions from each laboratory. These laboratory means can then be analyzed by a one-way ANOVA for the instrument parameter.

The ANOVA of the mean laboratory moments for the instrument parameter yielded no significant effect of instrument on the molecular mass distribution. The variation within instrument type was not significantly less than the variation among instruments. When the bins were examined by this method, only bin 8 was significantly influenced by the instrument parameter. Bin 8 represents 7% of the MMD and represents the high-mass tail of the distribution. Overall, the instrument has little influence on the molecular mass distribution of polystyrene.

Effect of Different Matrixes on the MMD. For the analysis of the sample preparation, only the laboratories that ran both dithranol and retinoic acid as matrixes were considered in the analysis. There were six laboratories that ran both matrixes.

A two-way ANOVA was performed on the data. The test first accounted for laboratory effects and then assessed the influence of the matrix on the moments and bins of the molecular mass distribution. The instrument parameter is also considered in this statistical method, because the instrument parameter is accounted by the laboratory parameter.

The two-way ANOVA results revealed that the matrix used in the sample preparation did not significantly influence the moments of the molecular mass distribution. When the bins were analyzed, only bin 3 was significantly influenced by the matrix parameter. Bin 3 includes data in the low-mass tail of the polymer molecular mass distribution and represents 6% of the MMD. The matrix used in sample preparation does not have a significant effect on the moments of the molecular mass distribution but may have an effect on the low-mass tail of the polymer distribution.

Effect of TOF-MS Mode on the MMD. The mode parameter indicates whether the TOF-MS was run in linear mode or reflectron mode. The mode is also confounded in the laboratory parameter. Not enough nonconfounded data are available to make a statement of effect of TOF-MS mode.

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7. DISCUSSION

The M_w and M_n obtained by MALDI-TOF-MS for the interlaboratory comparison were found to be 6.74 and 6.61 ku, respectively, with a standard deviation of 0.11 ku for M_w and 0.12 ku for M_n. The M_w of polystyrene determined by light scattering was found to be 7.19 ku with a sample standard deviation of 0.14 ku and an estimated expanded uncertainty of 0.56 ku. The M_n obtained by end group analysis by NMR for the polystyrene sample was found to be 7.05 ku with an estimated standard uncertainty of 0.40 ku.

We observe that the MALDI-MS gives lower M_n and M_w than do the classical methods. The estimated expanded uncertainties of the classical methods encompass the M_n and M_w averages obtained by MALDI-MS. However, the estimated expanded uncertainty of the classical methods includes systematic uncertainty evaluated as a type B uncertainty. The MALDI-MS data do not have an estimated systematic uncertainty. In fact, much of the research directed at MALDI-TOF-MS is aimed at estimating and lowering the systematic uncertainty evaluated as a type B uncertainty. However, it is noteworthy that the M_n and M_w values obtained by MALDI-MS in every laboratory that participated in the interlaboratory comparison are all lower than the M_n and M_w obtained by classical methods. The largest value of M_n reported was still 200 u less than the M_n obtained from NMR. The disagreement of the M_n and M_w obtained by MALDI-MS and the classical methods may be too great to be attributed to instrumental and statistical uncertainties alone, particularly since the bulk of the uncertainty in NMR and light scattering arises from very different sources. This, combined with the fact that the standard statistical uncertainty from the MALDI is so small, leads to the possibility that the systematic uncertainties in MALDI-TOF-MS may be biased in one direction. (The uncertainties from the classical methods are considered in ref 17.)

With this in mind, in the following paragraphs we consider the possible causes of systematic uncertainties that may arise in MALDI. Although we are unable as yet to put numerical values on the magnitude of these uncertainties, we shall consider whether we can determine the direction of the bias for some of the systematic uncertainties. The following discussion does not contain new theories; it is a discussion of possible causes of the uncertainties, which may explain the fact that the MALDI interlaboratory data seem to be consistently lower than classical values. Uncertainties arising from sample preparation, ablation and ion attachment, drift, detection, and data analysis are considered in the discussion.

Uncertainties Arising from Sample Preparation. There have been discussions of problems arising from sample preparation, specifically the use of solvent in which the polymer or matrix was not soluble to the concentrations expected or the use of mixed solvents, in which polymer may phase separate out of a solution.[24,25] These problems were not expected for protocol 1 or most of the protocols that the participants used for the unspecified protocol. The required protocol had been used by a number of the steering committee members with good success for PS of many various molecular masses. The only caution mentioned was to obtain the retinoic acid in a relatively pure form and keep it refrigerated, to prevent degradation. Fresh solutions of matrix and added salt are also required for the protocol as nonfresh solutions were found to yield poor results. In one case, a laboratory was not able to get signal following protocol 1.

Uncertainties Arising from Ablation and Ionization. Little is known about the desorption/ionization process in MALDI. Work on the same polymers of different molecular masses suggests that higher ablation energies are required for polymers of higher molecular masses. Thus, since it is normal procedure to use the ablation energy not too far above the threshold to obtain the polymer spectra in MALDI, the desorption process of MALDI could also cause the moments determined by mass spectrometry to be lower than the true moment. It is not known whether low-mass molecules desorb better than higher mass molecules, but if preferential desorption of low-mass molecules occurs, it could account for the low MALDI values.[5,25−27]

Little is known also about the ion attachment process with respect to molecular mass. However, from a naive statistical point of view, the larger the molecule, the more likely the attachment of the ion (more sites). This is at least consistent with multiple charging of synthetic polymers being seen at only higher molecular masses. It has been shown with very small synthetic polymers that the polymer molecule surrounds the cation, causing preference for a larger polymer.[28,29] The preferential attachment of the ions to larger polymers would cause an increase in the polymer mass.

Finally, there is the possibility of matrix salt or solvent attachment in addition to ion attachment to the polymer. Matrix salt attachment has been seen in synthetic polymers[20,30,31] while solvent attachment has been seen in some mass spectrometry of biopolymers.[32,33] We have seen that matrix salts are often very weakly attached and can be seen in postsource decay (PSD) spectra.[20] So far, our data indicate these effects are very small in synthetic polymers. If matrix or solvent adducts are seen in either linear or reflectron, they would cause an apparent mass increase.

Uncertainties Arising from the Drift Region. One obvious issue is the secondary peaks seen in Figure 3. These peaks were attributed to two causes. One cause was matrix attachment as discussed earlier. The other cause of the secondary peaks indicated some loss of styrene was occurring from the PS main chain. The moments of the molecular mass distribution would be affected by this fragmentation, causing values of the moments less than the actual values for the PS. When the impact of the fragmentation seen in Figure 3 was estimated, it was found not to be significant enough to cause the difference in M_n and M_w

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values that is seen between the MALDI determinations and classical methods,[20] although this fragmentation may contribute to the decreased mass we see. Furthermore, if PSD fragmentation were a major source of uncertainty, we would expect to see significant variations between the linear and reflectron modes, and that is not seen in the interlaboratory data (see section 5).

Uncertainties Arising from the Detector. The detector is another influence on the molecular mass distribution that may impact the moments calculated by MALDI-TOF-MS. The detector in most of the instruments is a microchannel plate (MCP) detector. The smaller molecular mass molecules hit the detector first and then the larger molecules last; if there is saturation occurring in the detector, then there would be a discrimination against the high-mass molecules. The issue of detector saturation has been discussed many times.[34,35] Since the matrix molecules or low molecular mass polystyrene molecules saturate the detector before the higher molecular mass molecules, this would cause the computation of a lower M_n and M_w than the true values.

The detector may also be less sensitive to larger mass polystyrene molecules due to the ion detection of the detector. MCP detectors count the number of ions by an ion-to-electron conversion when the ionized polymer collides with the detector plate. A bias occurs against the high-mass species when the ion-to-electron conversion is diminished due to decrease in the impact velocity of the larger ionic species.[35,36] If a discrimination against the high-mass polystyrene molecules exists, this would cause the moments of the distribution to be less than the true moments.

Uncertainties from Data Analysis. Baseline correction may also be a cause of the disagreement between the moments determined by classical methods and those determined by MALDI. There is much more noise in the low-mass end of the distribution obtained by mass spectrometry. Some of this noise is due to clusters of matrix and salt, but in general, there is more baseline noise in the low-mass areas of the distribution. When the baseline is corrected, does the correction account for this difference in sensitivity, and does this then have an impact on the integration of the peaks? If the baseline correction does not account for this difference in baseline noise, then the M_n and M_w calculated from the MMD will be lower than the true moment.

Uncertainties also can be caused with the integration of the MMD. If low- or high-mass signals are excluded from the integration, large variances can occur in the calculated moments. With respect to this particular uncertainty, we were able to reanalyze the data sets from one of the outlying laboratories. The molecular mass distributions of one outlying laboratory contained no data in bins 9−11, and this was the only laboratory to have no data in bin 9. We postulate that the integration software used was set by the experimenter to eliminate the small fragment peaks of the polystyrene, which also eliminated the high-mass tail peaks from the integration. The exclusion of data from the high-mass tails caused the moments to be extremely low, demonstrating the great significance and influence of proper integration of peaks. A uniform method of polymer molecular mass distribution integration is needed to eliminate these problems.

8. CONCLUSIONS

The M_w of interlaboratory comparison polystyrene determined by light scattering was found to be 7.19 ku with a sample standard deviation of 0.14 ku and an estimated expanded uncertainty of 0.56 ku. The M_n obtained by end group analysis by NMR for the polystyrene sample was found to be 7.05 ku with an estimated expanded uncertainty of 0.40 ku. The uncertainty estimates for M_w and M_n obtained by light scattering and NMR included both repeatability, a type A evaluation of uncertainty, and systematic uncertainties, a type B evaluation of uncertainty.[18] The M_w and M_n obtained by MALDI-TOF-MS for the interlaboratory comparison were found to be 6.74 and 6.61 ku, respectively, with a standard deviation of 0.11 ku for M_w and 0.12 ku for M_n.

NMR and FT-IR analysis confirm that the polystyrene has only one pair of end groups, as expected from the method of polymer synthesis. This is consistent with MALDI-MS run at NIST. Bottle-to-bottle variability on the polystyrene material sent out for the interlaboratory comparison was found to be below detectable levels by size exclusion chromatography.

The ANOVA data show that the variation among participating laboratories is significant. The type of instrument used in obtaining the MMD has little influence on the data. The matrixes that are used in the sample preparation of the polystyrene for MALDI-MS-TOF analysis do not have a significant influence on the molecular mass distribution. The mode of the instrument, linear or reflectron, contained insufficient data for analysis.

This analysis suggests that the mean value of the M_n and M_w moments from MALDI agree with those from light scattering and NMR within the level of estimated expanded uncertainty of the latter two methods. No estimation of overall uncertainty for MALDI-MS was possible since an estimate of systematic uncertainties, evaluated as a type B uncertainty, for MALDI-MS has not been made at this time. However, we made notice that the M_n and M_w from MALDI-MS reported by all participating laboratories are below the moment measurements made by the classical methods. With this in mind, we consider many possible systematic uncertainties from MALDI-MS and argue that many of them would favor MALDI-MS-obtained moments to be lower than classical methods. In all, we conclude this interlaboratory study shows that, under the conditions of a well-controlled protocol, MALDI-TOF-MS and classical methods agree for this polystyrene material.

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APPENDIX A: PARTICIPATING LABORATORIES

Botten, David S.; Lab Connections Inc.
Carmen, Howard S.; Eastman Chemical Company
Goldschmidt, Robert; Polymers Division, NIST
Hanton, Scott D.; Air Products and Chemicals, Inc.
Haverkamp, J.; Faculty of Chemistry, Utrect University, Bijvoet Center for Biomolecular Research
Mehl, John; Hercules, David; Chemistry Department, Vanderbilt University
Jackson, Tony; Molecular Spectroscopy Team, ICI Technology
Ji, Hellen; Mays, Jimmy; Department of Chemistry, University of Alabama—Birmingham
Kowalski, Paul; Bruker Daltonics
Lattimer, Robert; BF Goodrich
Li, Liang; Department of Chemistry, University of Alberta
McCarley, Tracy, Department of Chemistry, Louisiana State University
McEwen, Charles; Dupont Corporate Center for Analytical Sciences, Central Research & Development
Montaudo, Maurizio; Department of Chemistry, CNR—Institute for Chemistry and Technology of Polymeric Materials
Powell, Brian; Analytical Sciences Group—Spectroscopy, Zeneca Specialties
Price, Phillip C.; Union Carbide Corp., Central Research and Development
Schmidt, M.; Maskos, Michael; University Mainz
Wallace, William; Polymers Division, NIST
Weidner, Steffen; BAM—Federal Institute for Material Research & Testing (Berlin)
Weil, David A.; 3 M Corporate Research Labs
Wetzel, Stephanie; Girard, James E.; Department of Chemistry, American University, and Polymers Division, NIST
Wilkerson, Charles W., Jr.; Analytical Chemistry, Los Alamos National Labs
Wu, Kuang Jan; Analytical Research, Charles Evans & Associates

APPENDIX B: LABORATORY PROTOCOLS

Protocols 1 and 2 are given below.

INSTRUCTIONS FOR PROTOCOL #1

IN PROTOCOL #1 THE MATRIX, SALT, AND SOLVENT ARE PRESCRIBED

Name of Laboratory and Identifier sent to you:

Name of Principal Participant in Interlaboratory Comparison:

Name of Instrument Operator:

Name of Instrument, or if home built, please offer a brief description or include a copy of the paper in which the instrument is described:

Describe the sample preparation you used (matrix, salt, solvent, matrix concentration, salt concentration, hand spotting or electrospray, etc. Any and all other details would be useful.):

Describe instrument parameters (extraction field, laser energy, linear or reflectron detector used, detector type used, etc.):

Describe mass calibration:

For MALDI participants please use the following recipe:
5 mg/mL of PS in THF
75 mg/mL retinoic acid in THF
5 mg/mL AgTFA in THF
mix solutions by volume 1:10:1 of PS : retinoic acid : AgTFA

Please make three different sample spots, if you can, and take each spectrum from a different spot. If you can make only one spot, please take each spectrum from different area on that spot. (It is required that only one solution of solvent, polymer, matrix and salt be prepared with several sample spots mad from this one solution.) An individual spectrum should be at least 100 laser pulses. Do not change laser or machine settings during the time you make all 3 spectra. (You may want to make 4 spots so you can get the best machine setting to get the best spectra before you begin.)

Each participant will send me a separate ASCII file with M_n, M_w, and data as mass versus integrated peaks for each spectra.

Please make sure the peaks are integrated in time space. If they are integrated in mass space, certain corrections are necessary. (The corrections may be unimportant for the narrow MMD we are using here.)

The files sent to me should be of the form:

Use the following file name convention: LabXX_PS12.dat, where XX is the number assigned to your laboratory (which is the number of the tube you received), PS refers to polystyrene, 1 refers to the protocol number and 2 refers to the repeat in that protocol (thus PS12 means polystyrene, protocol #1, repeat #2).

First line: An identifying line having file name, your name, and your institution name.

Second line should be computed M_n for the repeat: e.g. 7248

Third line should be computed M_w for the repeat: e.g. 7307

Fourth line and on, mass of peak, integrated signal for the peak: 4040.0, 5.3
All other lines should be of the form: 6070, 13.7
Last line: end of data

I do not necessarily need commas, although if you can put them in that would be helpful, nor do I need "end of data" line. Obviously there must at least be a space between each number on each line if there are no commas.

INSTRUCTIONS FOR PROTOCOL #2

IN PROTOCOL #2 YOU CHOOSE THE MATRIX, SALT, AND SOLVENT

Name of Laboratory and Identifier sent to you:

Name of Principal Participant in Interlaboratory Comparison:

Name of Instrument Operator:

Name of Instrument, or if home built, please offer a brief description or include a copy of the paper in which the instrument is described:

Describe the sample preparation you used (matrix, salt, solvent, matrix concentration, salt concentration, hand spotting or electrospray, etc. Any and all other details would be useful.):

Describe instrument parameters (extraction field, laser energy, linear or reflectron detector used, detector type used, etc.):

Describe mass calibration:

For MALDI participants please described recipe used:

For other participants: Use your best technique and tell us what you did.

Please make three different sample spots, if you can, and take each spectrum from a different spot. If you can make only one spot, please take each spectrum from different area on that spot. (It is required that only one solution of solvent, polymer, matrix and salt be prepared with several sample spots mad from this one solution.) An individual spectrum should be at least 100 laser pulses. Do not change laser or machine settings during the time you make all 3 spectra. (You may want to make 4 spots so you can get the best machine setting to get the best spectra before you begin.)

Each participant will send me a separate ASCII file with M_n, M_w, and data as mass versus integrated peaks for each spectra.

Please make sure the peaks are integrated in time space. If they are integrated in mass space, certain corrections are necessary. (The corrections may be unimportant for the narrow MMD we are using here.)

The files sent to me should be of the form:

Use the following file name convention: LabXX_PS12.dat, where XX is the number assigned to your laboratory (which is the number of the tube you received), PS refers to polystyrene, 1 refers to the protocol number and 2 refers to the repeat in that protocol (thus PS12 means polystyrene, protocol #1, repeat #2).

First line: An identifying line having file name, your name, and your institution name.

Second line should be computed M_n for the repeat: e.g. 7248

Third line should be computed M_w for the repeat: e.g. 7307

Fourth line and on, mass of peak, integrated signal for the peak: 4040.0, 5.3
All other lines should be of the form: 6070, 13.7
Last line: end of data

I do not necessarily need commas, although if you can put them in that would be helpful, nor do I need "end of data" line. Obviously there must at least be a space between each number on each line if there are no commas.

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