Anal. Chem. **2005,** *77,* 4539−4548

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry Interlaboratory Comparison of Mixtures of Polystyrene with Different End Groups: Statistical Analysis of Mass Fractions and Mass Moments

Charles M. Guttman,* Stephanie J. Wetzel,† Kathleen M. Flynn, Bruno M. Fanconi, David L. VanderHart, and William E. Wallace

Polymers Division, National Institute of Standards and Technology, 100 Bureau Drive, Gaithersburg, Maryland 20899-8541

A matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) interlaboratory comparison was conducted on mixtures of synthetic polymers having the same repeat unit and closely matching molecular mass distributions but with different end groups. The interlaboratory comparison was designed to see how well the results from a group of experienced laboratories would agree on the mass fraction, and molecular mass distribution, of each polymer in a series of binary mixtures. Polystyrenes of a molecular mass near 9000 u were used. Both polystyrenes were initiated with the same butyl initiator; however, one was terminated with −H (termed PSH) and the other was terminated with $-CH_2CH_2OH$ (termed PSOH). End group composition of the individual polymers was checked by MALDI-TOF MS and by nuclear magnetic resonance (NMR). Five mixtures were created gravimetrically with mass ratios between 95:5 and 10:90 PSOH/PSH. Mixture compositions where measured by NMR and by Fourier transform infrared spectrometry (FT-IR). NMR and FT-IR were used to benchmark the performance of these methods in comparison to MALDI-TOF MS. Samples of these mixtures were sent to any institution requesting it. A total of 14 institutions participated. Analysis of variance was used to examine the influences of the independent parameters (participating laboratory, MALDI matrix, instrument manufacturer, TOF mass separation mode) on the measured mass fractions and molecular mass distributions for each polymer in each mixture. Two parameters, participating laboratory and instrument manufacturer, were determined to have a statistically significant influence. MALDI matrix and TOF mass separation mode (linear or reflectron) were found not to have a significant influence. Improper mass calibration, inadequate instrument optimization with respect to high signal-to-noise ratio across the entire mass range, and poor data analysis methods (e.g., baseline subtraction and peak integration) seemed to be the greatest obstacles in the correct application of MALDI-TOF MS to this problem. Each of these problems can be addressed with proper laboratory technique.

Over the past decade, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become a common technique for the analytical chemist working in the field of synthetic polymers.[1−12] Its ability to determine chemical composition as a function of molecular mass is of widespread practical interest.[13−17] In particular, low-mass homopolymers with different end groups are often studied because of their use as reactive prepolymers in a wide array of industrial processes. Furthermore, by determining end group composition as a function of reaction time (and, therefore, of molecular mass)

* Corresponding author. E-mail: charles.guttman@nist.gov.

† Present address: Department of Chemistry, Duquesne University, Pittsburgh, PA 15282.

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10.1021/ac050298h Not subject to U.S. Copyright. Publ. 2005 Am. Chem. Soc.
Published on Web 05/26/2005

polymerization processes can be better understood.[18] However, much is still unknown about the repeatability and the quantitative accuracy of MALDI-TOF MS applied to these types of problems. Under what conditions can it be relied upon to give a quantitative determination of the amount of, and the molecular mass distribution of, polymers possessing different end groups in a given mixture? What steps can the analytical chemist take to ensure quantitative results?

There are many aspects of the MALDI process that, if left uncontrolled, may prevent the attainment of quantitative results for a given mixture. Anything that allows for a detection bias as a function of end group composition or of polymer molecular mass will jeopardize the analysis. These include aspects of sample preparation, molecular desorption, molecular ionization,[19,20] mass separation, and ion detection. The report on our prior interlaboratory comparison[21] discusses some effects of these experimental factors in the study of the molecular mass distribution of a simple homopolymer. One method to obtain a measure of the overall robustness of a technique is to test each of these factors separately. This is a major thrust of the current work in our laboratory. Another method is to compare results on the same material between a number of experienced laboratories. To this end, the National Institute of Standards and Technology (NIST) initiated this interlaboratory comparison to learn more about polymer end group quantitation in MALDI-TOF MS as currently practiced. The interlaboratory comparison was designed to see how well a group of laboratories could get agreement on the percentages of each end group found in a mixture homopolymers. We aimed to identify the parameters that most influence quantitation of end group composition and molecular mass distribution and to compare the results from MALDI-TOF MS to those obtained from nuclear magnetic resonance (NMR) and Fourier transform infrared spectroscopy (FT-IR). We have chosen to study mixtures of polystyrene, a common and easy-to-handle polymer, each initiated with butyl initiator but terminated with either a polar ($-CH_2CH_2OH$) or nonpolar ($-H$) end group. In the rest of the paper these polymers are referred to as PSOH and PSH, respectively. This system was chosen for an interlaboratory comparison because we[13] and others[14] have found measurement conditions where the quantitation of mixtures of polystyrene seems possible. Five mixtures of these were made up with gravimetric mass ratios between 95:5 and 10:90 PSOH/PSH. This paper describes the MALDI-TOF MS analysis results of the 14 institutions (listed in the Acknowledgment) that participated in the interlaboratory comparison, as well as our own NMR and FT-IR work to quantify independently the end group percentages.

The outline of this paper is as follows. The synthesis of the polymers and preparation of the mixtures is described in Materials and Sample Preparation. We describe the experimental work done at NIST to quantitatively measure the end group composition of the starting materials and the composition of the mixtures by NMR in NMR Determination of Relative Amounts of PSOH Polymer in the As-Received Material and in Each Mixture. In FT-IR Determination of Relative Amounts of PSOH Polymer in Each Mixture, we describe the FT-IR work done at NIST to determine the relative amount of each polymer in the mixtures. A description of our protocol for the MALDI-TOF MS work to be done by each participating institution is given in MALDI-TOF MS Interlaboratory Protocol. The statistical analysis of all the data is given in Results: Description of the Complete Data Set. The effects of various measurement parameters such as matrix material are described in Results: Effect of Measurement Parameters on the Mass Fraction and Molecular Mass Distribution Estimates, with particular regard to the differences found between, and within, participating institutions. Finally, in Discussion, we draw some conclusions, and in Recommendations give a list of recommendations based on our findings.

MATERIALS AND SAMPLE PREPARATION

Synthesis. The PSH and PSOH used in this interlaboratory comparison were made by anionic polymerization and were obtained from commercial sources. For the PSH, two polymers of closely matched number-average molecular mass (M_n) and mass-average molecular mass (M_w) were used such that together they would span the complete mass range of the PSOH material used. From the preparation chemistry provided by the suppliers, we expected the PSH polymers to be atactic, have a narrow polydispersity, and be of the form

$$(CH_3)_2\text{-}CH-CH_2-[CH_2-CHPh]_n-CH_2-CH_2-PhH \qquad \text{Ph = phenyl} \quad (1)$$

For the PSOH, from the preparation chemistry provided by the supplier, we expected the polymer to be atactic, have a narrow polydispersity, and be of the form

$$(CH_3)_2\text{-}CH-CH_2-[CH_2-CHPh]_n-CH_2-CH_2-OH \qquad \text{Ph = phenyl} \quad (2)$$

Due to the difficulty in producing complete end group functionalization of the PSOH, it is expected that some amount of (1) or some amount of

$$(CH_3)_2\text{-}CH-CH_2-[CH_2-CHPh]_n-CH_2-CH_2-O-CH_2-CH_2-OH \qquad \text{Ph = phenyl} \quad (3)$$

may be present. An initial study by MALDI-TOF MS at NIST indicated that ~6% of (1), and very little if any of (3), were present in the as-supplied PSOH material. An earlier study[13] indicated that (3) was very easily observed by MALDI-TOF MS, giving us confidence that it really is absent from the as-supplied PSOH material.

Since the commercially supplied hydroxy-terminated polystyrene material contains ~6% proton-terminated polystyrene, shown in ref 1, we must be careful to distinguish in the text between "as-received PSOH *material*" and the specific "PSOH *polymer*" itself as shown in ref 2. In the same fashion, we shall refer to

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Table 1. Fraction of PSOH Polymer in Each Mixture As Prepared Gravimetrically, and Subsequently Determined by FT-IR and by NMR[a]

mix	gravimetric	FT-IR	NMR
A	1	1	0.995
B	0.7702	0.76	0.785
C	0.5167	0.502	0.514
D	0.3058	0.318	0.306
E	0.0927	0.106	0.093

[a] Uncertainty estimate for the gravimetric preparation less than 1% for each mixture, for the FT-IR a maximum uncertainty of 2.5% for mix E, and for NMR an uncertainty of ~2% for each mixture

"as-received PSH material" to distinguish it from specific "PSH polymer" as shown in ref 1.

It should be noted that the as-received PSOH material, even though supplied as a coarse powder, was found to have a significant within-bottle variation for its molecular mass distribution (MMD). This within bottle inhomogeneity is discussed in more detail in Supporting Information, Appendix B.

Sample Preparation and Bottling. Since the as-received PSOH material was inhomogeneous in the bottle, and two as-received PSH materials were used, the samples for the interlaboratory comparison were prepared by first making up weighed solutions of each type of material in weighed amounts of toluene and then mixing the two solutions in different proportions to obtain the mixtures used for the interlaboratory comparison. The solutions were arbitrarily designated mix A−mix E. An aliquot of each solution was put into a marked glass vial and the solvent evaporated away. For the interlaboratory comparison, 45 sets of 5 sample vials each were prepared. From the measured masses of toluene and polymer, the mass ratio of the as-received PSH material to the as-received PSOH material is given in the Table 1. This is not of course the ratio of the amount of PSOH polymer and PSH polymer in each of the solutions, since NMR (discussed next) and MALDI-TOF in our laboratory determined that there is ~6% PSH in the as-received PSOH material. Notice mix A is the as-received PSOH material alone.

MALDI-TOF MS was done at NIST to determine the polydispersity (PD) of each of the polymer components of the mixture and of the 50:50 mixture itself. We recognize that the PD determined by MALDI-TOF MS will be lower than that determined by other methods. The PSOH had a PD of 1.01, the PSH had a PD of 1.03, and the 50:50 mixture had a PD of 1.02. The estimated uncertainty in all PDs is 0.02. We did not use this value for any calculations in the paper but instead wished to reassure ourselves that the polymers were of narrow enough polydispersity to be amenable to MALDI-TOF MS analysis.

NMR DETERMINATION OF RELATIVE AMOUNTS OF PSOH POLYMER IN THE AS-RECEIVED MATERIAL AND IN EACH MIXTURE

NMR Sample Preparation. Samples, consisting of ~20 mg of polystyrene in toluene were transferred in a glass pipet directly into the NMR tube where they were then evacuated at ambient temperature to remove toluene. Following this, the samples were placed in a vacuum oven for 18 h at 90 °C, including two or three cycles where air was bled into the oven for more effective purging of toluene. During evacuation, a cold trap at liquid nitrogen temperature was used. Perdeuterated chloroform ($CDCl_3$) was used as the NMR solvent; samples were simply capped with the usual NMR plastic cap. A second sample of mix A was prepared using perdeuterated benzene (C_6D_6) as the solvent. This latter sample was sealed in air at a pressure slightly below ambient. Sample concentrations, by mass, were ~2.5% for the $CDCl_3$ solutions and ~4% for the C_6D_6 solution.

NMR Methodology. High-resolution, 270-MHz proton NMR spectra were taken on the samples. Bloch decays following single-pulse excitations were accumulated with pulse repetition times of 24 s and a pulse width corresponding to a 45 ° nutation angle. These parameters were chosen in order to obtain quantitative spectra. The observation temperature was 25 °C for the $CDCl_3$ solutions and 40 °C for the C_6D_6 solution. Between 100 and 200 scans were averaged. The spectral width of the Fourier transformations was 15 ppm, and the number of complex points in both the free induction decay (FID) and the Fourier transformed spectra was 16 384. Up to 0.2-Hz exponential line broadening was applied to the FIDs in order to minimize spectral ringing from data truncation. Careful baseline correction routines were applied in order to facilitate integration of smaller peaks.

NMR Data Interpretation. The integrals of interest that are most precisely determined are those of the aromatic protons (6.3−7.3 ppm) and the methylene protons of the hydroxymethyl terminus (3.1−3.5 ppm). The aromatic integrals are easily corrected for spurious contributions from $CHCl_3$ (on the downfield edge of the aromatic band) and from toluene (whose methyl integral at 2.36 ppm is easily evaluated and is used to calculate the corresponding integral correction for the aromatic region). The ratio, $R(x)$ for x = A−E, is that of the hydroxymethyl integral to the corrected aromatic integral, determined for each mixture. Since mix A is the component that is mixed with as-received PSH material for generating the other mixtures (B−E), $R(x)/R(\mathrm{A})$ is an excellent approximation to the mass fraction of mix A in the other mixtures. Those values are listed in Table 1.

The other integral of interest (0.5−0.8 ppm) is that of the six methyl protons on the initiating isobutyl terminus. All molecules possess one such termination. This integral is less precisely determined owing to partial overlap with the tail of the strong PS-methylene resonance (near 1.43 ppm) and overlap with another closer, but weak resonance near 1.0 ppm that is probably associated with the methylene protons of the isobutyl moiety. Accurate determination of the methyl integral would allow us to establish two other quantities of interest, namely, the fraction of molecules with hydroxymethyl terminals (proportional to the ratio of the hydroxymethyl integral to the methyl integral using the MALDI-TOF-MS-justified assumption that no molecules have more than one ethoxy group at a given teminus) and the overall average M_n (nearly proportional to the ratio of aromatic to methyl integrals).

It was deemed important to try to get the best possible assessment of the methyl integral for mix A, since mix A was a constituent of all the other mixtures. Hence, we also prepared a sample of mix A in perdeuterated benzene. In this solvent, both interfering resonances are moved downfield by ~0.1 ppm relative to the methyl resonance; hence, the methyl integral can be obtained more precisely compared with the case where $CDCl_3$ is

the solvent. Based on the integrals obtained from this sample, the fraction, $f_{OH}(A)$, of PSOH polymer in mix A associated with −OH ends is (0.94 ± 0.02) and M_n = (9040 ± 200) u. Values for $f_{OH}(x)$ can then be calculated for the other mixtures. Values of M_n for samples B−E are not determined; these values depend on the methyl integrals, which are not as accurately determined for the $CDCl_3$ mixtures. Incidentally, low molecular weight impurities associated with solvent-borne impurities or residues associated with the PS samples themselves generally show up as very sharp lines on top of the broader PS resonances. These features were absent in the regions of interest for integration or, in the cases of $CHCl_3$ and toluene cited earlier, were easily and accurately corrected for.

FT-IR DETERMINATION OF RELATIVE AMOUNTS OF PSOH POLYMER IN EACH MIXTURE

FT-IR Sample Preparation. The as-received PSH material and the homogenized as-received PSOH material, as well as each of the mixtures, were used as samples for the FT-IR analysis. Approximately 150 mg of dry KBr powder was mixed with the contents of a sample vial containing ∼7 mg of each material, transferred to a mortar for grinding, and then pressed into a pellet.

FT-IR Methodology. The oxygen−hydrogen, OH, bond stretch vibration was used as the measure of the hydroxyl-terminated polystyrene in the samples. This vibration absorbs in the infrared at ∼3592 cm^{-1}. Preliminary to the measurements on the interlaboratory comparison series, infrared measurements were made on both constituents in an attempt to determine the fraction of molecules in the as-received PSOH material that were not terminated by OH. It was not possible to determine M_n of the PSOH polymer owing to the absence of an infrared band attributable to the non-OH end group. Also, no reliable estimate of hydroxy-terminated content could be made based on the NMR-determined M_n owing to the lack of a reliable integrated extinction coefficient for the OH stretch vibration. Hydrogen bonding of the OH group, either with water or other OH groups, contributes to the uncertainty of the extinction coefficient. Even at dilute OH concentrations, as in the neat as-received PSOH material sample, a fraction of the OH groups participate in hydrogen bonding with other OH groups. The occurrence of hydrogen bonding shifts the OH stretch vibration frequency to lower energy and lowers the absorbance at the peak frequency of "free" OH group. For these reasons, measurements on the polystyrene mixtures were used only to determine OH content relative to that of as-received PSOH material. Relative measurements still require an independent determination of the amount of styrene. The integrated intensity in the region 1635−2000 cm^{-1} was used as the measure of styrene content. The weakly absorbing bands in this frequency region yield intensities that are within the range where Beer's law is applicable under the sampling condition used in the analysis. Content of as-received PSOH material in each mixture was determined from both integrated peaks and peak intensities of the OH band.

The infrared spectrum of non-oxygen-containing polystyrene exhibits several low-intensity combination or overtone bands in the 3500−370-cm^{-1} region. The spectrum of non-OH-terminated polystyrene was used to eliminate these styrene bands in the mixtures' spectra by spectral subtraction. In addition, liquid water absorbs at 3737 cm^{-1}. In the presence of small amounts of water, the OH-terminated polystyrene samples reveal a broad band in the 3300−3600-cm^{-1} region. Except for as-received PSOH material and mix A, the principal source of liquid water was from the KBr matrix. Spectral results from prepared KBr pellets were discarded if a band appeared at 3737 cm^{-1} that was more than 10% of the polystyrene OH band at 3592 cm^{-1}.

FT-IR Data Interpretation. Spectral comparison of the as-received PSOH material spectrum with that of mix A revealed that these two samples are indeed the same. Hence, the spectral results of mix A were used to determine the relative amount of hydroxy-terminated polystyrene in all other samples. In the spectra of both mix A and as-received PSOH material, a broad band appears in the 3300−3600-cm^{-1} region indicative of hydrogen bonding. In addition, the spectrum of mix A exhibited a weak liquid water band of integrated intensity ∼2% of that of the 3592-cm^{-1} band. The broad band indicative of hydrogen bonding did not appear in any other mixture's spectrum unless the liquid water band at 3737 cm^{-1} also appeared. Owing to the slight overlap of the broad H-bonded band with the 3592-cm^{-1} band, an attempt was made to remove the contribution. This was accomplished by subtracting the H-bonded band from the spectrum of mix A. The H-bonded band was estimated by assuming the band shape was symmetric about its peak frequency and that no other band(s) contributed to the observed intensity from the peak maximum to the lower frequency limit. The integrated and peak values of the 3592-cm^{-1} band of mix A were used to estimate the PSOH polymer content of each of the other samples. Table 1 summarizes the results achieved using the following data analysis operations: (1) The spectrum of water vapor was used to remove contributions of differences in water vapor between the background (empty sample compartment) and sample. (2) The spectrum of a non-OH-terminated polystyrene, corrected for water vapor, was used to remove by spectral subtraction the interfering styrene bands in the region of the OH stretch band. (3) Each spectral value in the OH stretch spectrum divided by the integrated intensity of the absorbance between 1635 and 2000 cm^{-1}. (4) The percentages reported in the peak intensity column of Table 1 were found by dividing the peak maximum intensities by that of mix A.

MALDI-TOF MS INTERLABORATORY PROTOCOL

The protocols for the interlaboratory comparison were decided upon by a steering committee organized from the membership of the Polymeric Materials Interest Group of the American Society for Mass Spectrometry. Each participating laboratory was asked to perform MALDI-TOF mass spectrometry using two protocols. The different protocols involved different sample preparations. The first of the protocols was specified. This protocol requires *all-trans*-retinoic acid for the matrix and silver trifluoroacetic acid for the salt.[10] The second protocol allowed each participant to use a sample preparation of their own choosing. Each laboratory was asked to produce two MALDI-TOF mass spectra for each protocol in order to check for intralaboratory variability. Each participant who performed both protocols would provide 10 spectra from the 5 mixtures with 2 samples preparations per mixture yielding 20 spectra total. Each laboratory was asked analyze their data in order to provide the mass ratio (see below for a detailed definition), the M_n of the PSH polymer, and the M_n of the PSOH polymer for each mixture for each repeat of the experiment, as well as the integrated mass intensity signal for each separate peak of the mass

spectrum with the cation mass subtracted from the peak masses. The mass ratio, the M_n of the PSH polymer, and M_n of the PSOH polymer for each mixture were also obtained through our analysis of integrated signal peak intensities reported by the participants.

RESULTS: DESCRIPTION OF THE COMPLETE DATA SET

Designation of Laboratory Numbers And Replication Numbers. As usual with any report on an interlaboratory comparisonn no institution is identified with any specific experimental result. Laboratory numbers were assigned randomly as samples were sent out. For each mix, between one and four sets of data were returned from each institution. Each data set was assigned an ordinal number (called a "repetition") for plotting and discussion purposes. For any measured or computed parameter discussed in this paper, a single value was assigned to each repetition.

Estimators of the Mass Fraction of Each Polymer Component in the Mixtures. The main focus of this interlaboratory comparison is the determination of the ratio of the masses of the two polymers in each of the five mixtures. We ask the participants compute this mass ratio from their data. This mass ratio is defined as the ratio of the concentrations of PSH polymer and PSOH polymer in the mixture. Thus, it can is computed as

$$\text{mass ratio} = \frac{\sum_i [m_i(\text{PSOH}) A_i(\text{PSOH})]}{\sum_i [m_i(\text{PSH}) A_i(\text{PSH})]}$$

where A_i(PSOH) is the area under the curve of the mass m_i(PSOH) associated with the PS with the OH end group and where A_i(PSH) is the area under the curve of the mass m_i(PSH) associated with the PS with the H end group. The same quantity can be obtained from the equations

$$\text{mass ratio} = \frac{M_n(\text{PSOH}) A(\text{PSOH})}{M_n(\text{PSH}) A(\text{PSH})}$$

$$A(\text{PSOH}) = \sum_i A_i(\text{PSOH})$$

$$A(\text{PSH}) = \sum_i A_i(\text{PSH})$$

The ratio of A(PSH)/A(PSOH), that is, the ratio of the total areas of the series with the H end group to those with the OH end groups, would, under ideal circumstances, give you the mole ratio of the total number of polymers with H and OH end groups, respectively. (MALDI-TOF MS is here assumed to measure the *number* of ions irrespective of their mass.) M_n(PSOH) is the number-average molecular mass of the series with the OH end group, and M_n(PSH) is the number-average of the polymers with the H end groups. Some participants assumed that by mass ratio we meant the ratio of M_n of PSOH polymer to the M_n of PSH polymer. This will of course not give the mass or concentration ratio for either of the species. These data were not included in the following discussions.

For all participants, when we analyzed their data, we used the software described in Appendix C (Supporting Information) to separate out the mass versus integrated peak area data into the series of PSH polymer and the series of PSOH polymer. From this we obtained our estimate of the mass ratio of the PSOH polymer to the PSH polymer and thus the mass fraction of each polymer in each of the mixtures.

Raw Data on Mass Fractions. We now turn to the main issue of this work, the agreement between the mass fractions estimated by NMR and FT-IR compared to those collectively estimated by MALDI-TOF MS. As we pointed out earlier in this paper, due to the fact that the as-received PSOH material was not functionalized to 100%, the comparison between the data of the mass fraction obtained by MALDI-TOF MS and that from the amount of mass of each as-received polymer we mixed together is a little difficult. By initial MALDI-TOF MS at NIST we found that there was ~6% PSH polymer in the as-received PSOH material. As described above, NMR gave values of 6% PSH polymer in the as-received PSOH material. The NMR estimate had a range of 4−8%.

We shall use the NMR estimate of 6% for the fraction of PSH in the as-received PSOH material as a means to estimate the fraction of PSOH polymer in mix A−mix E. Figure 1a gives the data from each of the laboratories for the fraction of PSH polymer in the as-received PSOH material, that is, in mix A. The squares represent the participants' calculation and the triangles are our calculation from their mass versus peak area data as obtained from our program described in Appendix C (Supporting Information). A line is drawn at 6% the best estimate of the NMR data of the fraction of PSH polymer in mix A and dashed lines are at 4 and 8%, that is, the extremes of the NMR estimate.

A number of other things should be noted for Figure 1a. First, generally when the participants did compute mass ratios, our estimate computed from their mass versus peak area data are in good agreement with a few notable exceptions discussed below. This indicates consistency in data analysis methods across participating laboratories. Second, comparing all the data with the NMR result, we find that ~1/3 of the data is above the upper limit estimated by NMR, ~1/3 below the lower limit estimated by NMR, and ~1/3 within the range of the data from NMR. This suggests the fraction of polymer measured by MALDI-TOF MS, even with its large laboratory-to-laboratory variation, is in good agreement with the NMR.

A few of the data require some specific mention. For one laboratory, repetitions 24 and 25, we computed the mass ratio from their data and found the fraction of PSH polymer in mix Al to be over 30%. This result is very high compared to the data from the other laboratories and from the NMR result. Data from repetitions 16−19, 36, and 37, coming from two different laboratories, each found no PSH material. In our recalculation using the software described in Appendix C of repetitions 16−19, we disagree with participants' calculations, while for 36 and 37 our calculation does indeed give no PSH polymer. In both cases, carefully looking at raw integrated data of masses versus peak areas shows consistency with our calculations.

We show the same plots for mix B−mix E in Figure 1b−e. For mix B, fewer points fall out of the NMR upper and lower limits (~15% fall out of upper and lower ranges) than do for mix A. Fewer fall out of the NMR range for mix C (~10%). However, in the plot

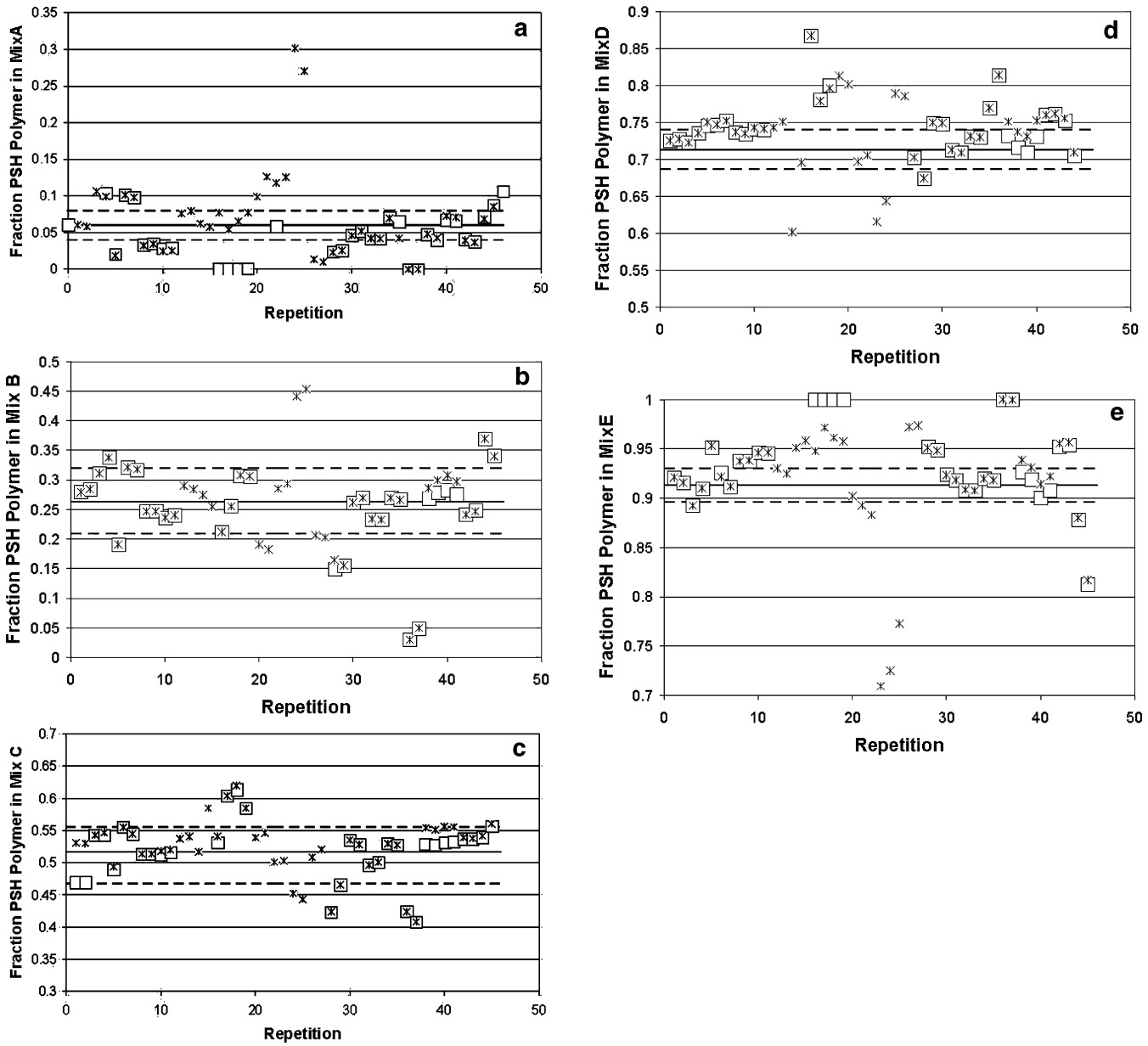

Figure 1. (a−e) Summary of all data received showing the MALDI-TOF MS determination of the fraction of PSH polymer for each mixtures. The squares are the participant's calculation of the fractions, and the triangles are NIST's calculations using their data. Notice that not all participants computed the mass fraction as requested. Each data set is assigned a ordinal number (called a "repetition") for plotting and discussion purposes.

for mix D and mix E, even more repetitions fall out of the range than in any of the other plots. We shall discuss this later.

Repetitions 24 and 25 are again outstanding in mix B and mix E. In mix A and mix B, they are too high, and in mix E, they are too low in PSH polymer. This all suggests that there was some leveling effect in the way the data were taken, all the data tending toward a 50:50 mixture. A detailed inspection of the data from repetitions 24 and 25 suggests that a baseline was incorrectly drawn (or more correctly said, a large baseline left on all the data) before the peaks were integrated, thus causing all the data to tend more toward a 50:50 mix. (A large offset on all the data would make all the data look like 50:50.) This baseline problem is often caused by the chemical noise arising from a large sweep down of the data before the polymer peaks. Repetitions 36 and 37 from the another laboratory showed perhaps too low measured PSH polymer in mix A and mix B and too high for PSH polymer in mix E. A result such as that from repetitions 36 and 37 may arise from poor signal-to-noise ratio (S/N) on the instrument. This suggests the experimentalist needs to optimize the instrument for S/N for the polymer/matrix sample preparation. Finally, for repetitions 16−19 on mix A and mix E, we do not agree with the calculations of the participant. The participant reports 0% PSH polymer in mix A and 100% PSH in mix E. Inspection of their mass versus area data does not show this. This indicates a data analysis problem.

Outliers. No outliers were removed from the data even though the laboratory providing repetitions 24 and 25 might be considered so because they apparently had misdrawn the baseline.

Mean Fraction of PSH Polymer and PSOH Polymer. In this section, we look at the mean mass fraction of PSOH polymer

Table 2. Mean Mass Fraction Of PSOH Polymer in Each Mixture As Determined by MALDI-TOF MS[a]

	mean fraction PSOH-polymer		
mix	gravimetric	MALDI mean participant's calcn	MALDI mean NIST's calcn
A	0.93	0.954 (0.031)	0.932 (0.057)
B	0.716	0.748 (0.073)	0.738 (0.077)
C	0.481	0.481 (0.045)	0.476 (0.043)
D	0.284	0.257 (0.036)	0.261 (0.048)
E	0.086	0.065 (0.040)	0.081 (0.059)

[a] Mass fraction of PSOH polymer assuming 6% PSH polymer in the as-received PSOH material using calculations provided by participants and calculations by NIST using the participants' integrated peak data. Uncertainty in gravimetric preparation less than 2% (0.02). Standard deviation of MALDI results listed in parentheses.

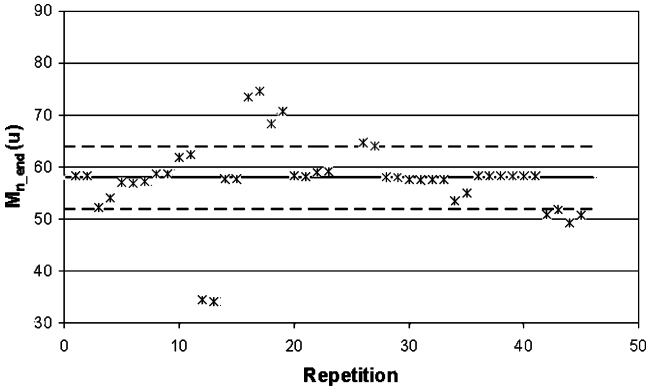

Figure 2. Mass-average molecular mass of the polymer end group, M_{n_end}, for the PSH polymer in mix C for different repetitions of the data.

in each of the five mixtures among all of the laboratories using all preparations and all of the data. The mean of the fractions, using their calculations and our calculations of each participant's data, is given in Table 2. The agreement between these two calculations is good owing to balancing of overestimates against underestimates across all participants. The agreement with the gravimetric results assuming a 6% PSH polymer in the as-received PSOH material (mix A) is shown in the Table 2. For the remainder of the mixes, we assume this 6% PSH polymer in the as-received PSOH material, mix A. All the data the column labeled gravimetric in Table 2 are the gravimetric values of the PSOH material corrected by the NMR data described above.

All the MALDI-TOF MS means are in good agreement with the uncertainties in the NMR and the uncertainties in the MALDI-TOF MS. Our calculations tend to be the same as the values obtained by the participants. The standard deviation is given for each of the five mixes in parentheses following each MALDI-TOF MS fraction. The agreement with the averages is good although the wide disparity of many of the data is reflected in the large standard deviation.

We did an analysis of variance (ANOVA) comparing the mean fraction of each polymer from the participants' calculation to those from our calculation using their data. We found for the five mixtures there was no difference between the means of the fractions calculated by us and by the participants as long as we excluded data from those laboratories where calculation errors were made (repetitions 16−19). If we include repetitions 16−19 in the means, the ANOVA shows the means calculated by us and the participants are different.

Mass Axis Calibration and Its Effect on End Groups and Series Estimation. As in our previous interlaboratory comparison,[21] although we asked that a mass axis calibration be done before the protocols were run, a number of the laboratories did a very poor job in calibration. It should be easy to calibrate to within a few mass units in this mass range for all instruments represented. In fact we received data in essentially three categories. Some laboratories did a careful job of calibration and reported to us their computed masses for each oligomer (we could tell this since the end groups and the repeat unit were the exact expected mass and no drift existed in the value of the computed end group). The majority of the laboratories did a fairly good job of calibration and although there was noise in the value of the end groups computed, they were within 2−3 u of the expected end group mass with little or no drifting of the computed mass. About 20% of the laboratories did a very poor job in calibration and obtained end groups off by as much as 20 u from the expected end group masses. Although their end group masses were erroneous, these authors had no trouble identifying which series was PSH polymer and which was PSOH polymer most likely due to their looking for mass differences of series locally.

Figure 2 gives the M_{n_end}, the number-average mass of the end group for the PSH polymer series mix C, as a function of repetition. The M_{n_end} was computed as

$$M_{n\_end} = \frac{\sum_i \left\{ \left[\left(\frac{m_i}{104.15} \right) - \mathrm{int}\left(\frac{m_i}{104.15} \right) \right] 104.15 A_i \right\}}{\sum_i A_i}$$

where A_i is the area assigned to mass m_i the mass of the oligomer, int() represents the greatest integer function, and 104.15 u is the average mass of the polystyrene repeat unit.

A horizontal line is drawn at the expected mass of the end groups, 58.1 u. These end groups were computed for the laboratories that did not tell us whether the silver cation mass was taken off from the masses reported in the table of masses versus areas each laboratory reported. Even if our assumption of this were incorrect, the end group would be off by no more than 4 u, the difference in mass between the average polystyrene repeat unit and silver. Yet in the plot we see many laboratories have M_{n_end} of more than 10 u. The dashed lines are offsets of ±6 u from 58 u. Still many fall outside this range.

In Figure 3 we plot the PD_{end}, the polydispersity of the measured end group mass. As usual with the normal PD, the PD_{end} is defined as

$$PD_{end} = M_{w\_end}/M_{n\_end}$$

where M_{w_end} is defined similarly to M_{n_end} above. As with the normal PD, the PD_{end} is related to the standard deviation of the distribution given by

$$PD - 1 = M_n^{-2}\{\overline{(m^2)} - (\bar{m})^2\}$$

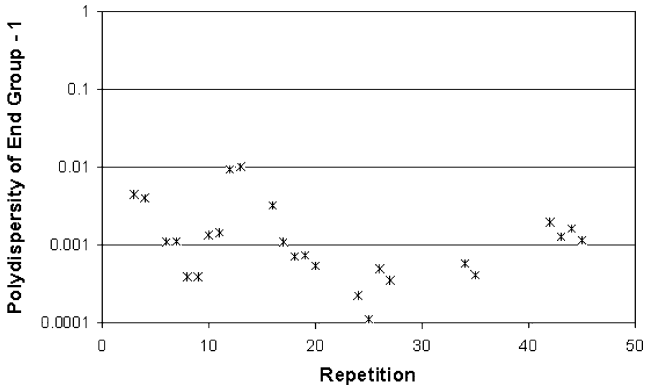

Figure 3. Polydispersity of the polymer end group, $PD_{n\_end,}$ for the PSH polymer in mix C for different repetitions of the data.

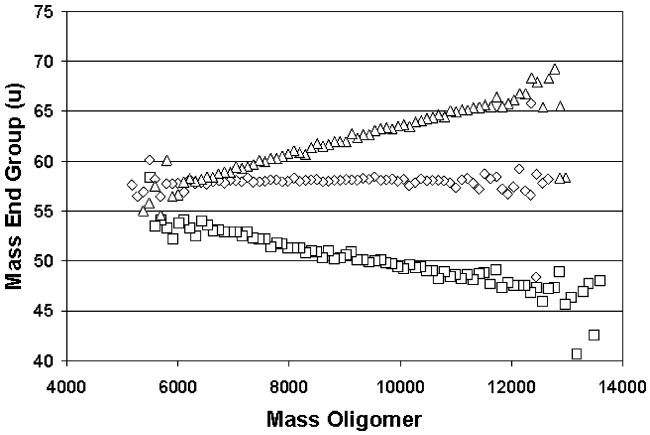

Figure 4. Computed end group mass versus oligomer mass for two polymer repetitions with poor calibrations (lines with finite slope) and one with a good calibration (line with zero slope) from mix C.

For end groups with a variance of 2 u and $M_n = 58$ u, we would estimate a PD_{end} of ~1.001. In Figure 3, the $PD_{end} - 1$ is plotted and there are a number of points out of the 1.001 range. These data show either high random noise on the mass axis (unlikely) or rather significant drift in the measured end group mass indicating poor calibration. In Appendix D (Supporting Information), we show the expectation of a poor calibration on apparent mass of the end group. In Figure 4 we have plotted the apparent mass of the end group versus m_i the mass of the oligomer for a few repetitions, which show poor calibration as determined by the wide PD of the end group mass. In addition, plotted is one data set, which shows outstanding calibration as determined by its narrow end group PD. A plot versus $m_i^{1/2}$, the square root of the mass of the oligomer, on a quadratic scale shows a no better fit of the data, indicating the leading term in eq D7 in Appendix D is dominant. Thus, we only show the linear in m_i plot.

Mean Moments of PSOH Polymer. The mean number-average molecular mass (M_n) of the PSOH polymer and for the PSH polymers for the entire data set, using all instruments and both protocols, is show in Table 3 for all five mixtures. The standard deviation (σ) for all mixes is given in parentheses for all of the M_n. In all of the PSOH polymers, the standard deviation is approximately equivalent in mass to ~1.5 repeat units of polystyrene except mix E, which has a much higher standard deviation. A much higher standard deviation is also seen in all the PSH polymer data. There seems to be no drift in the moments as we go to higher concentration of each species. Only at the lowest concentration of each species do we see a much lower M_n indicating perhaps a S/N problem.

Table 3. Number-Average Molecular Mass of PSOH Polymer and PSH Polymer in Each Mix[a]

mix	PSOH	PSH
A	8596 (125)	8350 (251)
B	8574 (130)	8601 (260)
C	8578 (157)	8641 (242)
D	8540 (156)	8638 (293)
E	8419 (265)	8654 (265)

[a] Values are in u. Numbers in parentheses are the standard deviation. Data averaged over all laboratories, all repeats, and both protocols.

RESULTS: EFFECT OF MEASUREMENT PARAMETERS ON THE MASS FRACTION AND MOLECULAR MASS DISTRIBUTION ESTIMATES

In the analysis of the interlaboratory comparison data, several parameters were considered as possible influences on the polystyrene fraction of either the PSOH polymer or the PSH polymer. The parameters examined were participating laboratory, sample preparation (i.e., matrix), instrument manufacturer, and TOF MS mode (i.e., reflectron or linear). Whether the laboratory in which the polymer was examined had an influence on the MMD is an important test of the robustness of the MALDI-TOF MS method. 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 in conjunction with protocol 2, but not by a great enough number of laboratories to make them statistically useful, so we were unable to include them in the comparison. The instrument parameter tests differences in the types of instruments. Three instrument manufacturers were represented: Applied Biosystems, Bruker Daltonics, and Shimadzu/Kratos. The parameter classifies by instrument manufacturer but not by instrument model for a given manufacturer. The mode of the instrument is tested to determine if there is an influence of mass separation mode, linear or reflectron.

Statistical Methods To Describe the Data. 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 experimental conditions, with the variation within experimental conditions. If the between experimental conditions variation differs greatly from the within experimental conditions variation, the means 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 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.

(22) Wallace, W. E.; Kearsley, A. J.; Guttman, C. M. *Anal. Chem.* **2004**, *76*, 2446−2452.
(23) Kachigan, S. K. *Multivariant Statistical Analysis*; Radius Press: New York, 1991.

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 Mass Fraction of PSOH Polymer. A one-way ANOVA of the mass fraction of PSOH for the parameter *laboratory* was performed on the data. The results showed that the parameter laboratory has a significant effect on the mass fraction of the PSOH polymer. But the one-way ANOVA of this parameter does not give conclusive results, because the parameter *instrument* and the parameter *laboratory* are confounded. Two parameters are confounded if their effects on the response variable, in this case the mass fraction, 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 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. In general, because of the confounding of the instrument and laboratory parameters, the data were reduced further to include only those instruments run by multiple laboratories. In our data, each instrument was run by at least two laboratories. 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 fractions of PSOH polymer

Effect of Instrument. The variable called instrument_manufacturer considers all instruments from the same manufacturer together as one parameter, regardless of the model of the instrument. There were three different instrument manufacturers identified in our study. Instrument from all three manufacturers were used by more than one laboratory, so all the laboratories were included in the analysis. Only protocol one data were considered in the statistical analysis.

To determine the effect of instrument_manufacturer on any data set, the parameter laboratory must be removed from the data set. This was achieved by taking the mean of the two repetitions of data from each laboratory. These laboratory means can then be analyzed by a one-way ANOVA for the parameter instrument_manufacturer.

The ANOVA of the mean laboratory moments for the parameter instrument_manufacturer yielded a significant effect of instrument on the fraction of the PSOH polymer for mixes B and C. When a two-way ANOVA was performed to eliminate the confounding of instrument and laboratory, the fraction of the PSOH polymer was found to vary significantly for all mixes. For both methods of analysis, the moments and end group masses were found not to vary significantly with instrument type. The variation of the mass fraction within instrument type was found to be significantly less than the variation of the mass fraction among instruments. Overall, the instrument has an influence on the mass fractions of PSOH polymer obtained.

Effect of Different Matrixes. Three different matrixes were used by the laboratories that participated in this round robin, dithranol, *all-trans* retinoic acid, and *trans,trans*-1,4-diphenyl-1,3-butadiene. A two-way ANOVA, to account for the laboratory effects, revealed an effect of matrix on the fraction of PSOH and the moments of the MMD. There was no significant variation of end group mass with matrix.

Effect of TOF-MS Mode. 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. A two-way ANOVA, which first accounted for the effects of laboratory, revealed no significant variation of the mass fraction with TOF-MS mode. This analysis did show significant variation of the moments of the mass distributions and end group mass with TOF-MS mode.

DISCUSSION

The average mass fraction of PSOH polymer obtained by MALDI-TOF MS for this interlaboratory comparison averaged over all participating institutions was found to be in good agreement with the NMR and FT-IR determinations for all mixtures. This result takes into account the 6% PSH polymer found in the as-received PSOH material. Further, MALDI-TOF MS, FT-IR, and NMR estimates of the amount of PSOH polymer in each of the mixtures were in good agreement with the gravimetric preparation results giving a satisfying overall consistency.

On the other hand, when the MALDI-TOF MS data are considered not in the aggregate but on an individual participant basis, MALDI-TOF MS showed significant laboratory-to-laboratory variation. Part of this could be accounted for by signal-to-noise differences between the laboratories. This suggests that not all participants took the time to optimize their instrument settings for the purpose of the interlaboratory comparison, or that their instrument could not be optimized sufficiently to see the peaks of the minority species above the noise. In addition, some of the variation could be accounted for by poor data analysis techniques.[22] Handling of data presented many participants with a significant challenge. Our reanalyzis of the participants' data using a consistent (though not necessarily the best) methodology went far in decreasing the differences between participants.

As in our previous interlaboratory comparison, we found a number of laboratories did a poor job of mass calibration on their instrument. This suggests that if they had to determine accurately the mass of the end groups, they would have been unable to. This problem was indicated by the dispersion among participants in the estimation of the M_{n_end} found for each of the polymers. This arose from two causes, either an incorrect (but unvarying across the spectrum) value for the end group mass or a drift in mass calibration across the spectrum causing a wider than expected polydispersity of end group mass.

In this work, we are somewhat surprised there was no effect of end group polarity of determining the proper mass fractions, or molecular mass distributions, in the mixtures due to such things as uncertainties arising from the cationization process. This may be due to the use of silver as the cationizing agent, which successfully competes against residual sodium and potassium found as impurities in the matrix. Silver is a better cationizing agent for polystyrene than sodium. Sodium and potassium in turn are better cationizing agents for poly(ethylene glycol) (PEG), while

silver is a poor cationizing agent for PEG. Since silver-cationized PS dominates the spectrum end group effects are not present.

RECOMMENDATIONS

Based on this and our prior interlaboratory comparison,[21] we make the following recommendations:

(1) Instrument mass calibration must be carefully performed. This is best done under measurement conditions as close to the analyte measurement conditions as is feasible. Biomacromolecules may not be the best choice for calibration when the analyte to be measured is a synthetic polymer due to the large differences in operating parameters required for these classes of sample.

(2) The instrument must be optimized for best signal-to-noise ratio in order to identify the minor components in a mixture. At this time, there seems to be no generally accepted, systematic procedure or set of necessary and sufficient criteria to optimize MALDI-TOF mass spectrometers. Most instrument tuning seems to be entirely dependent on the skill (and patience) of the operator.

(3) There is a need for generally accepted practices for data analysis, including, but not necessarily limited to, baseline subtraction, and peak integration. These procedures need to be supported by statistical theory in order that they may also provide meaningful uncertainties.

ACKNOWLEDGMENT

The authors thank all those who donated their time and expertise to participate in this interlaboratory comparison. For guidance in designing the MALDI-TOF MS protocol, C.M.G. thanks the members of the steering committee, Prof. Kevin G. Owens (Drexel University) and Dr. Scott D. Hanton (Air Products and Chemicals Corp.). C.M.G. thanks Dr. Stefan D. Leigh of the NIST Statistical Engineering Division for consultation on the statistical design of the interlaboratory comparison. the laboratories participating in this study are as follows: Academy of Sciences of the Czech Republic. Air Products and Chemicals Corp. (USA), Bruker Daltonics (USA), Bruker GmbH (Germany), Bausch & Lomb (USA), Bundesanstalt für Materialforschung und -prüfung (BAM) (Germany), Dow Chemical Co. (USA), Nagoya University (Japan), National Institute of Standards and Technology (NIST) (USA), National Institute of Advanced Industrial Science and Technology (AIST) (Japan), Shimadzu Corp. (Japan), University of Alberta (Canada), University of Alabama at Birmingham (USA), and University of Arkansas (USA).

SUPPORTING INFORMATION AVAILABLE

Addtional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review February 17, 2005. Accepted April 18, 2005.

AC050298H