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Interlaboratory comparison of average molecular mass and molecular mass distribution of a polystyrene reference material determined by MALDI-TOF mass spectrometry

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Abstract

To evaluate the robustness of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) for synthetic polymers, an interlaboratory comparison of molecular mass distributions (MMDs) measured by the method was carried out at the National Institute of Advanced Industrial Science and Technology (AIST), Japan. Ten participants performed measurements on identical samples of polystyrene (certified $M_w = 2423 \pm 20$) by using three different types of instrument at four different locations. Two-way and one-way analysis of variance (ANOVA) revealed that instrument settings and measurement protocols have more influence on the MMD than does the operator.

Keywords: Interlaboratory comparison; MALDI-TOF mass spectrometry; Average molecular mass; Molecular mass distribution; Polystyrene

1. Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) has become an important and essential analytical tool, especially in the field of protein research [1,2]. However, it has been pointed out that there are problems in the quantitative applications of mass spectrometry for the following reasons [3–6].

(1) The amounts of ions produced and the stability of the ions are markedly dependent on the length of the molecular chain. This effect is often referred to as the mass discrimination effect.
(2) When large numbers of low-molecular-mass ions impinge on the detector simultaneously, the sensitivity of the detector towards the higher-molecular-mass ions is reduced considerably as a result of the saturation caused by the low-mass ions.
(3) The number of generated electrons depends on the speed of the colliding ions in a multi-channel plate (MCP) detector and/or a secondary electron multiplier. This means that the sensitivity to large molecules is reduced, because the speed of the ions becomes slower as the mass that has to be accelerated by a constant voltage increases.

These issues are less crucial where the emphasis is on the quantitativeness of the signal intensity, for example, in proteome analysis. However, the issues are too important to be neglected for samples such as synthetic polymers that, by their nature, have a molecular mass distribution (MMD).

Despite these problems, MALDI-TOFMS is still expected to become an important method for determining the average molecular mass and MMD of synthetic polymers with relatively narrow MMDs ($M_w/M_n < 1.1$) [7–9]. In fact, very accurate average molecular mass values can be obtained by MALDI-TOFMS coupled with size-exclusion chromatography (SEC/MALDI-MS), even for polydisperse polymer samples. This new method has been proposed recently, and an algorithm has been established by one of the authors [10]. More specifically, one can obtain MALDI spectra that are apparently consistent over a wide mass range by measuring MALDI spectra for fractionated

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samples with M_w/M_n of less than 1.1 (prepared by SEC fractionation), and synthesizing the overall spectrum, which has accurate relative intensity ratios corrected by means of the area ratios determined from the SEC chromatogram. The advantages of this method are: (1) the composition of individual polymer molecules can be determined, and (2) an accurate molecular mass distribution can be obtained without any SEC calibration. In order to generalize this method, it is also important to evaluate the repeatability and accuracy of MALDI-TOFMS itself.

Recently, national research organizations, such as the National Institute of Standards and Technology (NIST) in the USA [11,12] and the Federal Institute for Materials Research and Testing (BAM) in Germany, have been conducting international interlaboratory measurements by using standard samples in order to evaluate the reliability of average molecular mass and MMD measurements obtained by MALDI-TOFMS. We also participated in these interlaboratory measurements, but we are planning full-scale independent interlaboratory measurements within Japan to evaluate MALDI-TOFMS quantitatively.

We had already tried a small-scale interlaboratory comparison as a preliminary experiment. Although we have described the details of this interlaboratory comparison in another report [13], we will outline it below.

Measurements on poly(ethylene glycol) (PEG; average molecular mass 5–50 kDa) and poly(methyl methacrylate) (PMMA; average molecular mass 2–100 kDa) were performed by three laboratories, one at the National Institute of Advanced Industrial Science and Technology (AIST) and two at private companies. These laboratories were asked to follow the procedures provided during the international interlaboratory measurement conducted by BAM. In this measurement, we designated 2,5-dihydroxybenzoic acid (DHBA) as a matrix and sodium trifluoroacetic acid (for PEG) or lithium chloride (for PMMA) as a cationization agent. We also prescribed the preparation method of sample spots, the measuring mode, and the accumulated number of measurements, etc. However, the measuring instruments were the ones that each operator used routinely. As a result, for both PEG and PMMA samples, there was only a 1–5% relative standard deviation among the operators when measuring the average molecular mass value. The precision or repeatability of the MALDI-TOFMS measurements was superior to other methods, such as SEC, which is generally used for determining molecular mass of synthetic polymers. Although significant differences were detected in the data reported, we could not determine whether these differences were related to the skills of the operator or to the different measuring instruments. In addition, it was found that any measured value was significantly biased against the reference values that were obtained by other methods. Therefore, it is very important to compare the average molecular mass measured by MALDI-TOFMS with that of certified reference material in order to evaluate the observed bias.

In this second preliminary interlaboratory study, we therefore focused on the effects of the parameters “instrument” and “operator” on MALDI results and the accuracy of the method. One of the characteristics of this interlaboratory measurement was that we used a monodisperse polystyrene ($M_w = 2423$) that had been characterized by supercritical fluid chromatography (SFC) and certified by the National Metrology Institute of Japan (NMIJ), AIST. The average molecular mass and MMD of the reference material are accurately known and certified, and the levels of uncertainty are given. This enabled us to compare the average molecular mass and M_w/M_n values obtained by MALDI-TOFMS with the certified one. The other characteristic of our interlaboratory comparison is that we used a standard statistical analysis tool called analysis of variance (ANOVA). In ANOVA, we compare means by splitting the overall observed variance into different parts. For example, if between experimental conditions variation differs significantly from within experimental conditions variation, we can conclude that means are not to be equal. In this study, we used three different types of commercial instruments and three different operators took measurements using all the instruments. In a preliminary experiment conducted in the first interlaboratory measurement, one-way analysis of variance (ANOVA) suggested that the results from different instruments (different laboratories) showed significant differences. In this report, we conducted a two-way ANOVA evaluation to simultaneously distinguish the effect of instruments from those of operators.

2. Preliminary discussion and study

The objectives, evaluation procedure, and protocols were discussed at preliminary meetings of a steering committee organized by the polymer mass members at AIST. Factors that could conceivably affect MALDI results were listed (Table 1). One of

Table 1
Factors to be considered in MALDI-TOFMS results

Factors	Causes of uncertainty
Instrument	Difference in manufacturer and type
Quality of sample spot	Differences in habit of operators in placing the spot on the plate Even if the method is regulated, deposited spots will show disparities
Laser energy	Subjectivity of the operator Energy will be regulated at a value slightly higher than threshold
Laser irradiation point	Differences in the point selected by the operators
Data processing method	Differences in data-processing software integral to the instrument
Calibration	Differences between multi-point and single-point calibration
Operating mode	Difference between linear and reflectron modes
Selection of the reagents	Differences in the matrix, salt, and solvent
Concentration of the reagents	Differences arising from inconsistencies in sample preparation
Quality of the reagents	Effect of impurities from reagents
Quality of the miscellaneous items	Effect of contamination from implements

the most important purposes of this interlaboratory comparison was to elucidate the influence of instruments and operators on the measured average molecular mass.

To this end, we initially fixed the operating mode (linear or reflectron), the reagents (matrix, salt, and solvent) and their concentrations and purities, and the quality of the pipette tips and bottles used. Then we enumerated other factors, classified by type of variation (independent variable or dependent variable). In this report, the following items were studied.

(1) The weight-average molecular mass (M_w) and number-average molecular mass (M_n) of the certified polystyrene reference material determined by MALDI measurements were evaluated by a two-way ANOVA study on "instrument" and "operator" to identify any significant differences among different instruments and operators.
(2) The effect of the operator's skill on the results was analyzed by ANOVA or significant difference inspection.
(3) The difference between the MALDI result and the SFC result (reference value) was evaluated by a significant difference inspection.
(4) The bias in the distribution of MMD measured by MALDI-TOFMS was evaluated by comparison between the results from MALDI-TOFMS and those from SFC (certified values).

For reference, the interlaboratory protocols chosen by the Steering Committee are given in Appendix A.

3. Experimental

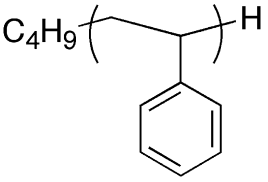
3.1. Instruments

Four commercial MALDI-TOFMS spectrometers of three different types, named X1, X2, Y, and Z (X1 and X2 are the same type) equipped with pulsed nitrogen lasers with a wavelength of 337 nm and a 3 ns pulse width were used in this study. Instruments used were listed below. They were operated in the linear mode with positive-ion detection and a high voltage of 20 kV.

Shimadzu, Kompact III.
Bruker Daltonics, Reflex III.
Applied Biosystems, Voyager DE-Pro.

3.2. Certified reference material (CRM) polystyrene PS 2400

A certified reference material (CRM) PS 2400 (NMIJ CRM 5001-a) was used as the measurement sample. This CRM is supplied by the National Metrology Institute of Japan, AIST. Because the polymer was prepared by anionic polymerization with butyllithium as the initiator, its chemical structure is expected to be:



This chemical structure was confirmed by ^{1}H and ^{13}C NMR.

The M_w, M_n, and M_w/M_n of PS 2400 as determined by SFC, with uncertainties, are:

$$M_w = 2307 \pm 18$$

$$M_n = 2423 \pm 20$$

$$M_w/M_n = 1.050 \pm 0.016$$

Number after the symbol ± is the expanded uncertainty with the coverage factor $k = 2$.

The molecular mass (M_i) of the ith component is calculated from the following equation:

$$M_i = 104.149i + 58.1220$$

where i is the degree of polymerization, and all the atomic weights of the elements are taken from the table published in 2001 by The International Union of Pure and Applied Chemistry [14].

3.3. Reagents and miscellaneous items

Identical reagents were used for sample preparation in all the measurements. 1,8-dihydroxy-9(10H)-anthracenone (dithranol, Sigma–Aldrich, Japan), antioxidant-free tetrahydrofuran (THF, Wako Pure Chemical Industries, Ltd.), and silver trifluoroacetate (AgTFA, Sigma–Aldrich, Japan) were used without further purification. Eppendorf® pipette tips (10, 100, and 1000 μL) and Pyrex® glass tubes with screw caps for mixing the reagents were distributed to all the operators.

3.4. Measurement protocols

All the operators were required to perform MALDI-TOFMS measurements by using the same protocol, i.e., protocol #1. In this protocol, participants were required to prepare their samples themselves using the reagents, pipette tips, and glass tubes described above. The second protocol, protocol #2, allowed some operators to use sample preparation or measurement conditions of their own choosing. The details of the two protocols are listed in Appendix A.

3.5. Structure of the interlaboratory comparison

All 10 operators (A–J) carried out MALDI-TOFMS experiments on identical samples of PS by using 4 commercial MALDI-TOF mass instruments of 3different types (X1, X2, Y, and Z). Three of the operators (A, B, and C) were experienced or skilled researchers who had been involved in MALDI-TOFMS

Table 2
Structure of the interlaboratory comparison

Category	Operators	Measurement symbol	Instruments and measurement protocols[a,b]			
			X1	X2	Y	Z
Skilled	A	A1	P1		P1	P1
		A2	P2		P2	
Skilled	B	B1	P1		P1	P1
		B2			P2	P2
Skilled	C	C1	P1		P1	P1
Novice	D	D1	P1	P1		
Novice	E	E1	P1	P1		
Novice	F	F1	P1	P1		
Novice	G	G1	P1	P1		
Novice	H	H1	P1	P1		
Novice	I	I1	P1	P1		
None	J	J1				P1
		J2				P2

[a] P1: protocol #1, P2: protocol #2.

[b] Instruments X1 and X2 are the same model.

experiments in their researches. They operated three instruments, i.e., X1, Y, and Z using protocol #1. In addition to these measurements, they also performed measurements using protocol #2. The other operators (D–I) were less experienced researchers; they operated only instruments X1 and X2 using protocol #1. Data from the operator J (classified neither "skilled" nor "novice") was used just for reference. An outline of the interlaboratory test is given in Table 2.

4. Results and discussion

4.1. Description of overall data

Fig. 1 shows typical spectra measured by using instruments X1, X2, Y, and Z. In general, all the spectra exhibited a series of ion peaks at m/z $104.1n + 166.0$, where 104.1 is the mass of the repeating unit of polystyrene, n is the number of repeating units, and 166.0 represents the residual mass of the end groups and adducted cation (Ag^+). Although all the spectra are similar to each other, many slight differences can be found. Influences of these easily missed differences will be analyzed in more detail later.

Figs. 2 and 3 show all the reported M_w and M_w/M_n values for PS 2400 measured by using protocols #1 and #2, respectively. The M_w values measured by all the operators using the instrument X1 fell below the certified M_w. Furthermore, for M_w/M_n, the instruments X1 and X2 also provided much smaller values. The reason why these instruments provide smaller M_w/M_n values will be discussed later. On the other hand, instruments Y and Z provided M_w and M_w/M_n values in good accord with the certified values.

It was also found that the inter-operator variations of M_w and M_w/M_n were very small for the same instrument and a similar measurement protocol. For example, the relative standard deviation of the M_w measured by all the operators using the identical instrument X1 was approximately 3%. On the other hand, a larger variation was found in M_w and M_w/M_n data measured using protocol #2, where matrixes different from those in protocol #1 were used, showing that the choice of matrix could have a significant effect on the MALDI results.

To distinguish or separate the effect of the parameter "instrument" on the MALDI results from that of the parameter

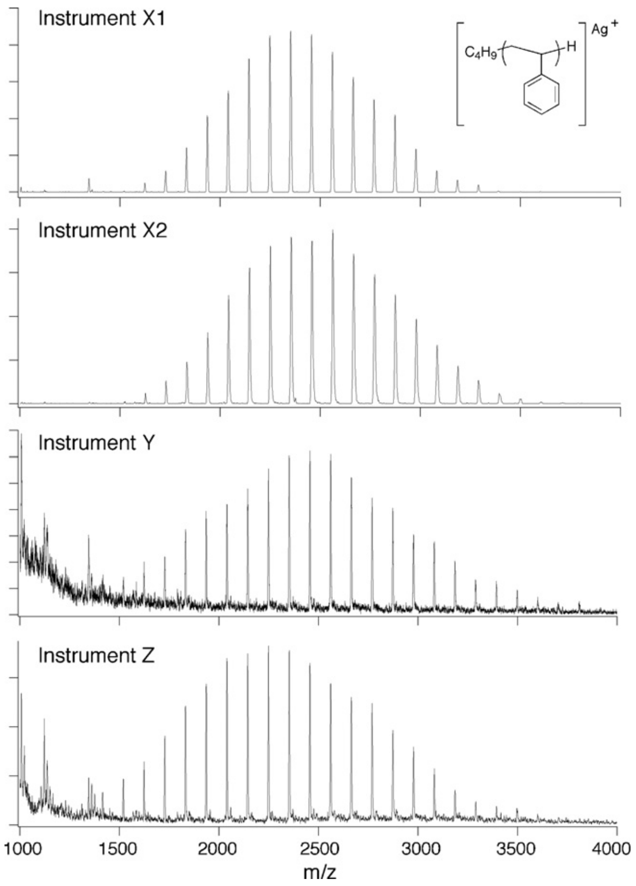


Fig. 1. Typical spectra measured using instruments X1, X2, Y, and Z.

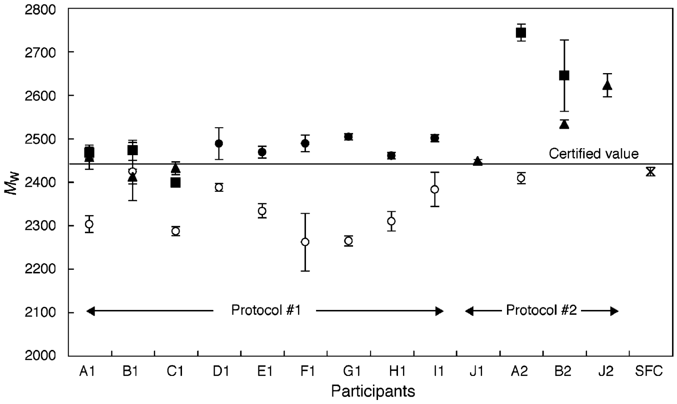


Fig. 2. Overview of measured values of the weight-average relative molecular mass M_w. (Error bars show the standard deviation for each value.) The horizontal line and the dotted line represent the certified M_w and its uncertainty, respectively. Instrument: X1 (○), X2 (●), Y (■), Z (▲).

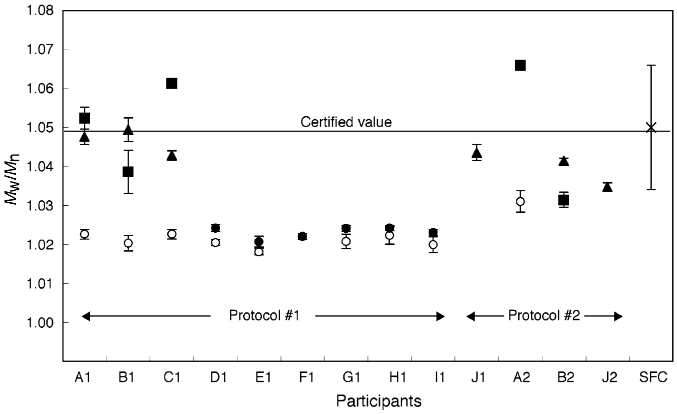


Fig. 3. Overview of the measured molecular mass distributions M_w/M_n. (Error bars show the standard deviation for each value.) The symbols are the same as for Fig. 2.

“operator”, a two-way ANOVA should be performed. In general, it is hard to perform the analysis completely because each operator should operate all the MALDI instruments with identical measurement protocols. Even in the interlaboratory comparisons conducted by NIST, they could not analyze a complete set of data [11,12]. For example, they could not distinguish the effect of instrument on the results from that of laboratory because their interlaboratory tests were not designed for each laboratory uses more than one type of instrument [11]. In this study, three skilled researchers measured the PS sample using one another’s instruments with the same protocol (protocol #1).

Table 3
Variations in the MALDI measurements evaluated two-way ANOVA[a]

	M_w	M_w/M_n
Repeatability[b]	±28 (±1.2%)	±0.003 (±0.3%)
Variation among the operators	±16 (±0.6%)	±0.002 (±0.1%)
Variation among the instruments	±52 (±2.1%)	±0.015 (±1.4%)

[a] Two-way ANOVA result from the values obtained by three skilled operators, three types of the instruments, and protocol #1.

[b] All the operators repeated measurements three times.

Statistically significant differences among operators and instruments were found for both M_w and M_w/M_n. This means that the two parameters “operator” and “instrument” can produce significant biases in MALDI measurement values. In particular, the comparison among instruments showed surprising differences. These results indicate that users of MALDI-TOFMS should understand that this technique could provide significant differences between different instruments.

To estimate the uncertainties arising from the instrument and the operator, we calculated repeatability, within-instrument variation, within-operator variation, and the combined uncertainty of the MALDI measurements. The two-way ANOVA results are summarized in Table 3. The M_w and M_w/M_n values measured by the three skilled operators (A, B, and C) using the three different types of instrument with protocol #1 were used for the analysis. As the table shows, the variation in repeatability among operators of the MALDI measurements was extremely small. Such good repeatability (±1.2% for M_w and ±0.3% for M_w/M_n) revealed significant differences between instruments or operators. On the other hand, even if we find these significant differences, MALDI can provide consistent or outstanding results with other measurement methods on preciseness [15,16].

Table 4 shows the average values of M_w and M_w/M_n for each instrument and operator using protocol #1. Although significant failures in M_w were observed on the instruments X1 and X2, all the collected M_w results are still close to the certified or true values. In particular, the values measured by instruments Y and Z are in very good accord with the certified M_w value within the range of uncertainty. In addition to M_w, it is noteworthy that M_w/M_n values measured by using the two instruments Y and Z agreed well with the certified M_w/M_n value. This indicates that MALDI-TOFMS has the potential to provide very accurate measurements of average molecular masses and MMDs.

The conclusions are as follows.

Table 4
Statistical results of weight-average molecular mass and molecular mass distribution for each instrument

	Instrument				Certified values
	X1	X2	Y	Z	
Number of operators	9	6	3	3	
Skill of operators	Skilled and Novice	Novice	Skilled	Skilled	
M_w	2328 ± 10	2485 ± 17	2446 ± 38	2433 ± 26	2423 ± 20
M_w/M_n	1.021 ± 0.002	1.023 ± 0.001	1.051 ± 0.010	1.047 ± 0.004	1.050 ± 0.016

(1) MALDI-TOFMS results greatly depend on the instrument, but do not depend on the operator's experience.

(2) In terms of the analysis of monodisperse polystyrene with a low molecular mass, MALDI-TOFMS gives very accurate and comparable average molecular mass and $M_\mathrm{w}/M_\mathrm{n}$ values if instruments are suitably conditioned and measurement protocols are appropriate to the samples to be measured. For example, the results obtained by skilled operators (instruments Y and Z were employed) are averaged as:

$$M_\mathrm{w} = 2440 \pm 30, \qquad \frac{M_\mathrm{w}}{M_\mathrm{n}} = 1.049 \pm 0.008$$

which agree closely with the certified values within uncertainties.

4.2. Effect of instrument

A glance at Table 4 and Fig. 3 shows that all the $M_\mathrm{w}/M_\mathrm{n}$ values given by the instruments X1 and X2 are considerably lower than the certified value (1.050 ± 0.016). The results are concentrated around 1.02. Additionally, instrument X1 provided lower M_w values, irrespective of the operator. It should be noted that differences in the M_w value are observed between instruments of the same type, i.e., between X1 and X2.

To investigate the effect of the instrument on the MALDI results in more detail, we performed a one-way ANOVA test of the parameter "instrument" including the M_w, M_n, or $M_\mathrm{w}/M_\mathrm{n}$ data taken by each skilled operator A, B, and C using the three different instruments. Each operator performed measurements repeatedly three times for each instrument. For each operator, the total number of the data to be analyzed were 9 for M_w, M_n, or $M_\mathrm{w}/M_\mathrm{n}$. The statistical results shows that the parameter "instrument" has a significant effect on $M_\mathrm{w}/M_\mathrm{n}$ irrespective of the operators. The ratio of the between instruments variance to the within instruments for $M_\mathrm{w}/M_\mathrm{n}$, i.e., the F-value, was found to be 53 much larger than the F critical value 3.4 for the degree of freedom of 2 and 24. The statistical results of M_w and M_n obtained by the two of the three operators showed that the parameter "instrument" also has a significant effect on these properties. For instance, the F-value for M_w measured by the operator A was 57 which was much greater than the F critical value of 5.1. These findings indicate that difference in the instrument type could give significant difference in measured average molecular weights and MMD.

When all the MALDI measurements had been performed, the members of the steering committee discussed why the results from instruments X1 and X2 showed significant differences from those of the other two instruments. Fig. 4 shows a comparison of the MMD measured by operator A with the certified $M_\mathrm{w}/M_\mathrm{n}$. The $M_\mathrm{w}/M_\mathrm{n}$ data measured by instrument Z agreed fairly well with the certified $M_\mathrm{w}/M_\mathrm{n}$ for the entire molecular mass region. However, no signals in the lower (n; 8–10) and higher (n; 35–40) molecular mass regions were observed with instrument X1. Operators B and C also reported similar results.

In the detector of instrument X, an offset device is arranged at the amplifier. If the offset level is too high, weak signals originating from analytes disappear as a result of noise around the baseline. To investigate the effect of the offset level on MALDI results, a simple check was carried out by using instrument X2. Initially, MALDI measurements were performed at high, medium, and low offset levels using protocol #1. At the high offset level, $M_\mathrm{w}/M_\mathrm{n}$ for the polystyrene sample was calculated to be 1.021 ± 0.006. This is almost the same as the result of the interlaboratory comparison. At the medium and low levels, however, values of 1.036 ± 0.002 and 1.043 ± 0.002 were found, respectively. Therefore, instrument X can give good results, close to the certified $M_\mathrm{w}/M_\mathrm{n}$ value (1.050 ± 0.016, $k = 2$), if the offset parameter is adequately adjusted. From the results of this test, it is clear that instruments X1 and X2 were adjusted to high offset level before shipping by the manufacturer. This observation also indicates that an inexperienced operator should notice the importance of setting appropriate measurement parameters.

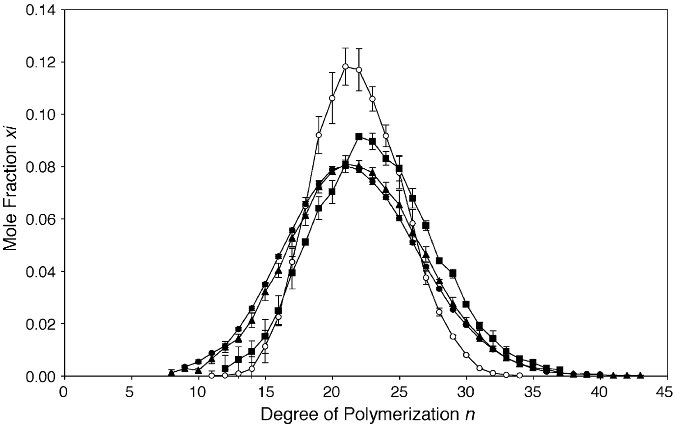


Fig. 4. Comparison of molecular mass distributions measured by operator A. Certified mole fraction (●), mole fraction determined by using instrument X1 (○), Y (■), and Z (▲).

The $M_\mathrm{w}/M_\mathrm{n}$ obtained by operator A with instrument Y also has the same problem in the lower molecular-mass region (n; 8–11, see Fig. 4); however, this did not happen for all the skilled operators. Instrument Y has two types of peak-cutting function to protect the detector from an overflow of low-molecular-mass ions. One function is "deflection", which deflects and disposes ions for a period of time from extraction. The other is the "suppression" system, which attenuates signals detected at earlier flight times. In this interlaboratory comparison, the selection of these functions was not regulated. Operator A set a deflection voltage of up to m/z 500. The influence of deflection extended at m/z values above 500, and decreased the number of ions around m/z 1000. After the interlaboratory comparison, operator A investigated the peak-cutting parameters. When the suppression was on at up to m/z 250 and the deflection was off, the measured spectrum reproduced almost the same $M_\mathrm{w}/M_\mathrm{n}$ as the certified one.

4.3. Effect of operator

To investigate the effect of the parameter "operator", we performed one-way ANOVA tests on the M_w, M_n, and $M_\mathrm{w}/M_\mathrm{n}$ data measured by different operators using each identical instrument.

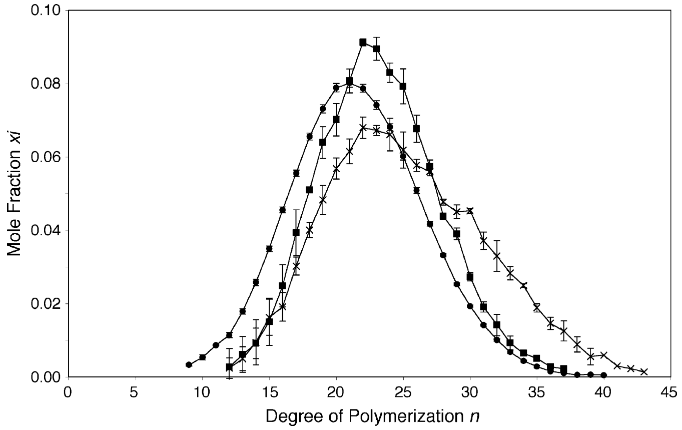


Fig. 5. Comparison between measurements A1 and A2 (molecular mass distribution measured by operator A with instrument Y). Certified mole fraction (●); mole fraction determined by using instrument Y with protocol #1 (■) and protocol #2 (✖).

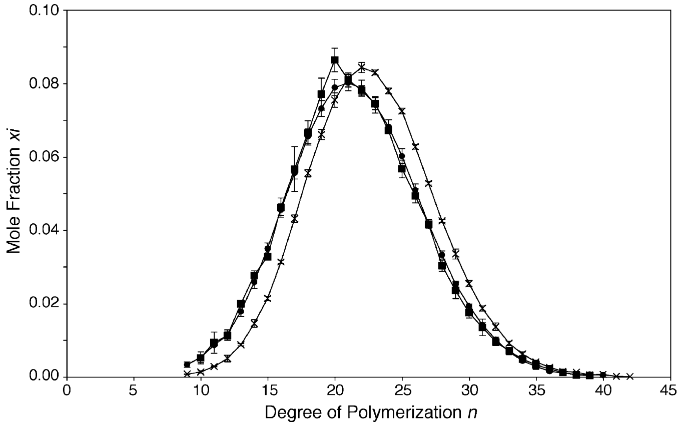


Fig. 6. Comparison between measurements B1 and B2 (molecular mass distribution measured by operator B with instrument Z). Certified mole fraction (●); mole fraction determined by using instrument Z with protocol #1 (■) and protocol #2 (✖).

For M_w, M_n, and M_w/M_n data, no significant effect of the parameter "operator" was observed for the instrument X1, X2, and Z. In particular, all operators using instrument X reported very similar M_w and M_w/M_n values, irrespective of their experience. An inspection by the steering committee found that this agreement arose from the fixed operating parameters of instrument X.

On the other hand, for instrument Y, significant effects were detected for both M_w and M_w/M_n. Detailed inspection by the committee revealed that the difference was attributable to the instrument settings, not to the operator's skill. As already shown in Fig. 4, the main cause of the disparity was a difference in the cutoff parameters of the instrument.

4.4. Effect of different matrixes

In protocol #2, the operators were allowed to choose the matrix, salt, solvent, and any other conditions freely. For example, operator A changed the matrix used in the protocol #1 to cobalt ultra-fine powder [17], keeping all the other conditions the same as in protocol #1, and performed the analysis using instrument Y (measurement A2). This measurement provided the highest M_w (2743.0) among all the reported values in this interlaboratory comparison. A comparison of the observed M_w/M_n values between A1 and A2 for the instrument Y is given (Fig. 5). As the figure shows, the M_w/M_n measured with the cobalt powder shows a longer high-mass tail than that measured with dithranol. Because identical measurement conditions, except for the matrixes, were used in both measurements, the longer tail can be attributed to the cobalt powder. Because low-background-noise spectra are obtained, it has been said that a cobalt matrix is effective for low-molecular-weight samples [1,17]. In this case, we have shown that this matrix has the ability to ionize larger molecules efficiently. This might be caused by a characteristic of inorganic fine particles, such as a rapid heating/vaporization process on UV laser irradiation.

Fig. 6 shows another example of the effect of changing matrixes. In these measurements, i.e., B2 and J2, 1,1,4,4-tetraphenyl-1,3-butadiene [18] was used as the matrix. This compound is a new matrix that has high ionization efficiency, particularly for low-polarity or nonpolar synthetic polymers; it also has the particular ability of maintaining its ionization efficiency for a long time (>60 min) under high-vacuum condition. Operator B used instruments Y and Z, and J used instrument Z. These results also show considerably higher M_w values (2532.4–2644.0) than those obtained by protocol #1. The results with protocol #2 show that it is necessary to fix the matrix in order to obtain accurate average molecular masses in polymer MALDI analyses. This fact only becomes apparent when we use a certified reference material as a sample for comparison.

4.5. Comparison between the results from MALDI and the other analytical methods

Recently, a careful evaluation and comparison for the accuracy of different molecular-mass determination methods was conducted by measuring a polystyrene certified reference material [19]. The M_n, M_w, and M_w/M_n values measured by SFC, SEC, SEC coupled with multi-angle light-scattering detection (SEC-MALS), and ^{1}H NMR are presented in Table 5. The values of SFC and SEC are in extremely close agreement with each

Table 5
Comparison of observed average molecular mass of PS 2400 between different molecular mass determination methods[a]

Method	M_n	M_w	M_w/M_n
SFC[b]	2307 ± 9	2423 ± 10	1.050 ± 0.008
SEC[b]	2292 ± 15	2415 ± 16	1.054 ± 0.001
SEC-MALS[c]	2265 ± 33	2522 ± 17	1.113 ± 0.013
^{1}H NMR[c]	2357 ± 2		
MALDI-TOFMS[d]	2326 ± 42	2439 ± 30	1.049 ± 0.008

[a] Data of SFC, SEC, SEC-MALS, and ^{1}H NMR are from reference 19. The SFC data are taken as certified values of PS 2400.

[b] Numbers after ± denote standard uncertainties.

[c] Numbers after ± simply denote experimental standard deviations for repeated measurements.

[d] Average values of the data obtained by the skilled operators using the instruments Y and Z with the protocol #1.

other, because the calibration curve used in the SEC measurements was constructed by using uniform polystyrenes, whose M_w/M_n is 1 and were fractionated by supercritical fluid chromatography [19]. If commercial reference materials were used for the calibration, we would expect less-accurate data for SEC.

We also show, for comparison, the values measured by operators A, B, and C using the three different MALDI instruments in this interlaboratory study. Note that MALDI-TOFMS can provide very accurate average molecular mass and MMD measurements compared with other methods, such as SEC-MALS and ^{1}H NMR. To the authors' knowledge, this comparison provides the first evidence that MALDI-TOFMS can provide correct MMD data.

5. Conclusion

To evaluate the robustness of MALDI-TOFMS for synthetic polymers, an interlaboratory comparison of MMD was carried out. Ten operators measured identical polystyrene samples ($M_w = 2423 \pm 20$) using three different types of instrument at four different locations. Two-way and one-way ANOVA data showed that both the instrument and the operator have significant effects on the average relative molecular masses and M_w/M_n. The effect of the instrument was found to be more significant than that of the operator. These findings indicate the importance of instrumental settings. The matrixes that are used in the sample preparation have a significant influence on the MMD.

Despite the significant differences between instrument and instrumental settings, MALDI-TOFMS has the potential to provide comparable data between different laboratories because the variations among them are relatively small. This indicates the possibility of producing measurement standards for the method. It is also noteworthy that MALDI-TOFMS has the ability to provide very accurate M_w/M_n values for monodisperse polymers. If we use SEC as a separation method to prepare fractional samples for MALDI-TOFMS measurements, we can evaluate MMDs for polydisperse and higher-molecular-mass polymers. To summarize, MALDI-TOFMS can achieve a greater precision and accuracy than the other molecular-weight analysis methods (e.g., SEC) if the instruments are suitably equipped and suitable instructions (protocols) are tightly obeyed.

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Appendix A

A.1. Interlaboratory protocol #1

All the operators are required to comply with the following protocol.

- *Polymer sample for the interlaboratory comparison*
  Sample name: polystyrene (PS) NMIJ CRM5001-a.
- *Reagents*
  Matrix: 1,8-dihydroxy-9(10H)-anthracenone (dithranol).
  Solvent: tetrahydrofuran (THF, no stabilizer, sealed in N_2).
  Salt: silver trifluoroacetate (AgTFA).
- *Miscellaneous matters*
  All the operators are required to use the same pipette tips and bottles.
- *Solution preparation*
  Solutions (A)–(C) are prepared separately.
  PS solution (A): PS in THF (1 mg/mL).
  Matrix solution (B): dithranol in THF (10 mg/mL).
  Salt solution (C): AgTFA in THF (1 mg/mL).

Mix solutions (A), (B), and (C) in the ratio 1:4:1 by volume just before recording the spectrum.

Once opened, the bottle of THF should be used immediately before it degrades.

- *Spotting procedure*
  Please deposit 1 μL or less of the mixed solution by hand spotting.
- *Mass calibration*
  Self-calibration at the peaks for the 15-, 21-, and 27-mers is required. Theoretical masses of the Ag-adducted peaks are:
  15-mer, $C_{124}H_{130}Ag$: 1728.1.
  21-mer, $C_{172}H_{178}Ag$: 2353.0.
  27-mer, $C_{220}H_{226}Ag$: 2977.9.

All these values are calculated as relative molecular masses (do not use monoisotopic masses). If the resolution of your instrument is too high, please smooth the peaks of the spectra before calibration.

- *Acquisition of the spectrum*
  Number of sample spots: 3.
  Number of spectra: 3 (take each spectrum from a different spot).
  Number of the laser pulses: 200 (for an individual spectrum).
  Laser energy: set slightly higher than threshold (the way to find the threshold is not regulated).
  Accelerating voltage: fix in an individual instrument.
  Operating mode: fix on linear mode.
  Data processing: use the software supplied with each individual instrument.
- *Things for submission required*
  Report: prepare on the prescribed form (see Appendix C).
  Electronic file: send ASCII files of each raw spectrum.

A.1.1. Calculation of the number of moles of molecules with a molecular mass of M_i (x_i)

Please refer to following equation:

$$x_i = \frac{A_i}{\sum_i A_i}$$

where A_i is peak area of M_i.

Please calculate by using the software supplied with the individual instrument or other suitable software.

Please fill in all x_i values and averages of three acquisitions in the blank form.

A.1.2. Calculation of the M_w, M_n, and M_w/M_n values

Please calculate by using the software supplied with the individual instrument or other suitable software.

Please fill in M_w, M_n, $M_\mathrm{w}/M_\mathrm{n}$, and averages of three acquisitions in the blank form.

A.2. Interlaboratory protocol #2

Skilled operators can choose the matrix, salt, solvent, and any other conditions freely.

Appendix B. Participating laboratories

- *Skilled operators*
  Sato, Hiroaki; Research Institute for Environmental Management Technology, AIST.
  Togashi, Hisashi; National Metrology Institute of Japan, AIST.
  Nagahata, Ritsuko; Research Institute for Innovation in Sustainable Chemistry, AIST.
- *Novice operators*
  Arasawa, Hiroko; Research Institute for Innovation in Sustainable Chemistry, AIST.
  Itakura, Masanao; National Metrology Institute of Japan, AIST.
  Unno, Akihiro; Hitachi Chemical Co., Ltd.
  Kishine, Kana; National Metrology Institute of Japan, AIST.
  Shimada, Kayori; National Metrology Institute of Japan, AIST.
  Matsuyama, Shigetomo; National Metrology Institute of Japan, AIST.
- *Nonclassified participant*
  Seino, Teruyuki; Research Institute for Environmental Management Technology, AIST.

Appendix C

All operators are required to submit their reports in the prescribed form as follows.

- *Basal data*
  Name of operator.
  Name of laboratory.
  Name of instrument.
  Protocol number.
  Date of experiment.
  Smoothing (“Yes” or “No”).
  Baseline correction (“Yes” or “No”).
  Delayed extraction (“Yes” or “No”).
  Accelerating voltage.
  Peak picking (“Area” or “Height”).
  Sampling rate (ns or Hz).
- *Average relative molecular mass*
  First line: M_n of three values and average of these.
  Second line: M_w of three values and average of these.
  Third line: $M_\mathrm{w}/M_\mathrm{n}$ of three values and average of these.
- *Number of moles of molecules*
  Table: i versus x_i of three respective values and average of these.
- *Comments*
  Any comments and other details would be useful.

Appendix D

The definition of the average relative molecular masses was as follows.

The molecular masses of the component with a degree of polymerization i are:

$$M_n = \frac{\sum_i x_i M_i}{\sum_i x_i}$$

$$M_\mathrm{w} = \frac{\sum_i x_i M_i}{\sum_i x_i}$$

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