
Quantitation of Synthetic Polymers Using an Internal Standard by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

Hui Chen and Meiyu He

Department of Chemistry, Peking University, Beijing, China

MALDI-TOF MS is utilized to perform quantitative analysis on synthetic polymers. Despite the inherent limitations of MALDI, good quantitative results have been obtained in the three sets of experiments described here. An internal standard with similar molecular properties as the analytes is introduced. Plots of relative integrated intensity ratios as a function of theoretical ratios of stoichiometry are drawn based on the results. The satisfactory slopes and correlation coefficients illustrated the practicality of quantitative measurement by MALDI-TOF MS. (J Am Soc Mass Spectrom 2005, 16, 100–106) © 2004 American Society for Mass Spectrometry

Since the work of Tanaka et al. [1] and Karas and Hillenkamp [2] in 1988, matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry has developed into a convenient and powerful technique for the analysis of large macromolecules, for instance, biomolecules [3–5] and polymeric compounds [6–10]. There are the properties that make MALDI-TOF MS a more useful tool than the other traditional analytic methods including UV absorption, circular dichroism, nuclear magnetic resonance, and light scattering [11]—the three “S” advantages of mass spectrometry: specificity, sensitivity, and speed, according to McLafferty’s opinion [12]. In addition, a fourth “S” advantage for MALDI-TOF MS should include spaciousness: the ability to analyze molecules across an extensive mass range. Although research has been focused on qualitative characterization of those macromolecules, the technique has gradually been applied to quantitative measurement: for biomolecules by Nelson and McLean [13] and Hercules et al. [14] in 1994 and for synthetic polymeric compounds by Gardella et al. [15] in 2002.

In the case of synthetic polymer quantitative analysis, much less effort has been made compared to that of biomolecules. In industry, many polymers are in fact copolymers which take advantage of the properties of two (or more) different classes of monomer. Therefore, the reliable method of measuring the fraction of each component precisely is crucial. However, some indigenous factors of MALDI hinder the quantitative analysis on synthetic polymers. The limitations arise mainly from factors such as the homogeneity of the sample/

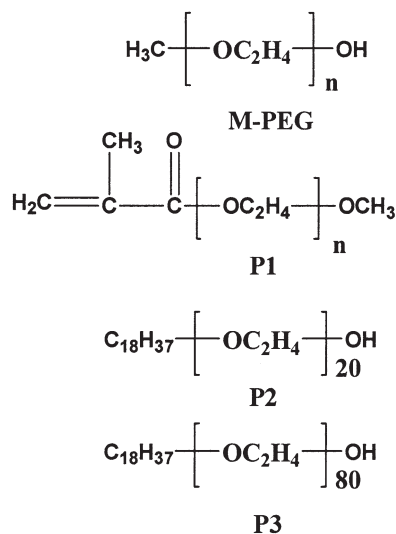
matrix/cationization salt spot on the target, which is considered to contribute to the notable instability of signal intensities of analytes in the spectra, the different ionization efficiencies and differences in desorption and detection of the different components which are concerned as the most critical parameter in determining the intensities of peaks in the MALDI spectra, the distribution of molecular weights of oligomer chains and subunits which causes the signal to appear more complicated in the mass spectra of polymer than the single peak in the case of biomolecules, the mass discrimination in the process of ionization, and transmission and detection which makes the intensities of the mass spectra lower both in low mass range and high mass range. Combined, these factors are believed to compromise the precision, reproducibility, and practicality with which quantitative measurement on synthetic polymers can be made.

Despite the limitations, some achievement has been accomplished in quantitation of synthetic polymers by MALDI-TOF. For instance, Gardella and his coworkers [15] reported quantitative MALDI-TOF measurements of polydimethylsiloxane of two different molecular weights. Murgasova and Hercules [16] carried out the quantitative characterization of a polystyrene/poly-methylstyrene blend using coupled size-exclusion chromatography and MALDI mass spectrometry. Additionally, in our previous study [17], we demonstrated the possibility of quantitative analysis of different components of a polymer sample. Two polyethylene glycols with different end-groups were regarded as different components of a simulated “polymer” through artificial mixture. The relative ion intensity ratios of two components were found to have quantitative relations with their ratios of moles.

Although the work above conducted the quantitative

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Address reprint requests to Prof. M. He, Department of Chemistry, Peking University, Beijing 100871, China. E-mail: myhe@pku.edu.cn



Structure 1. The structures of the synthetic polymers studied.

measurements on different study targets by different methods, none introduced the concept of an internal standard, which is promising to correct the vulnerabilities inherent to MALDI quantitative experiments. In the present study, we intended to investigate the feasibility of quantitative analysis of a fixed component in different polymer samples by using an internal standard. We designed three sets of experiments to carry out the quantitative determinations on synthetic polymers (P1–P3) with methyl polyethylene glycols (M-PEG) as the internal standard by MALDI-TOF MS. Chart 1 shows the structures of these polymers. Good linearity was observed in the plot related to the molecular ratio (x axis) and the signal intensity ratio (y axis) based on the data of all the three sets of experiments. The satisfactory results vividly illustrated the feasibility of quantitative analysis on synthetic polymers and the utility of the introduction of an internal standard.

Experimental

General Instrumentation and Conditions

MALDI experiments were carried out using a Bruker BIFLEX III time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Billerica, MA). The instrument is equipped with a N_2 laser emitting at 337 nm (Laser Sciences Inc., Cambridge, MA), a 1-GHz sampling rate digitizer, a pulsed ion extraction source, and an electrostatic reflectron. The laser pulse width is 3 ns and its maximum power is 200 μJ . Spectra were acquired in the positive-ion mode. The acceleration voltage was 19 kV. The delay voltage was 14.3 kV, the delay time 200 ns, and the reflectron voltage was 20 kV. Typically, 150 single-shot mass spectra were summed to give a composite spectrum. All data were reprocessed using the Bruker XTOF software. The mass scale was calibrated externally, using the peptides angiotensin II, bovine insulin b-chain, and bovine insulin as mass standards.

Samples and Reagents

The MALDI analysis of the synthetic polymers utilized dithranol (DI) (Aldrich, Milwaukee, WI) as the matrix. The solvent, inhibitor-free tetrahydrofuran (Aldrich), stored in a Sure/Seal bottle, was used as received. NaCl was used as the cationization agent (Acros, Fairlawn, NJ). Trifluoroacetic acid (TFA) and methanol were bought from Acros. M-PEG, P1, P2, and P3 were used as received (Aldrich, Milwaukee, WI). The polymers M-PEG, P1, and P2 were dissolved in THF with the concentration of 1 mg/mL while P3 of 3 mg/mL. The matrix DI was also dissolved in THF at a concentration of 10 mg/mL. The concentration of NaCl solution was 5 mg/mL using a mixed solvent ($\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{TFA}$, 2:1:0.03). The detailed mixing ratios of the polymers studied in three sets of experiments are shown in Table 1. M-PEG was used as the internal standard with the fixed mole. To maintain the constant concentration of M-PEG in the mixture, THF was added to keep the total mixture volume at 31 μL . The mixture was treated under ultrasonic conditions for 3 min to ensure the samples were well-mixed. We used the dried droplet method to prepare the sample target: 1 μL of analyte mixture was applied to the sample target and air-dried.

Results and Discussion

Characterization of Synthetic Polymers

The properties of synthetic polymers are largely dependent on the structures of polymers, the molecular weight distribution, and the proportion of each component. Therefore, the qualitative characterization of synthetic polymers is as important as the quantitative analysis for full characterization on synthetic polymers. The qualitative characterization of polymers includes determining the molecular weight distribution, calculating the mass of the repeat-unit, and deducing the structures of end-groups from the oligomer masses, which can be easily accomplished in good MALDI spectra. However, to obtain good MALDI spectra is not trivial. The proper selection of matrix [18], cationization agent and solvent [19], the mixing ratio of analyte, matrix and cation, and the laser power utilized, Yan et al. [15], are crucial for the MALDI experiments. An earlier study [20] showed that dithranol is the best

Table 1. Ratios of the analyte mixtures of set A–C

Sample	DI (μL)	M-PEG (μL)	Pn ^a (μL)	THF (μL)
1	20	4	1	6
2	20	4	2	5
3	20	4	3	4
4	20	4	4	3
5	20	4	5	2
6	20	4	6	1
7	20	4	7	0

^an = 1–3.

Table 2. Molecular weights of the synthetic polymers

Polymers	Mw	Mn	PD (Mw/Mn)
M-PEG	800	780	1.03
P1	960	920	1.04
P2	1200	1160	1.03
P3	3660	3320	1.1

matrix for PEGs. For the consistency of all the spectra, NaCl was used as the cationization salt. From the MALDI spectra under linear mode, we can calculate the average molecular weights of the synthetic polymers studied. The observed weight average (Mw) and number average (Mn) of polymers **M-PEG** and **P1-P3** are shown in Table 2.

The Mw and Mn have equal importance in the quantitation. In fact their ratio, the polydispersity, is also important. Mw and Mn are used for different things because they correlate to different properties. In our study, the number of polymer moles is calculated by Mn because it relates more to the number.

Another critical parameter is the composition of end-groups. In our study, the masses of end-groups are necessary for the calculation of peak intensities of integrated oligomer distributions in the MALDI spectra (see eq 2). According to eq 1 (where M_{end} represents the mass of end-group, M_{peak} represents the mass of the selected peak, M_{cat} represent the mass of cation, and $n \cdot M_{\text{re}}$ represents n times the mass of the repeat unit),

$$M_{\text{end}} = M_{\text{peak}} - M_{\text{cat}} - n \cdot M_{\text{re}} \quad (1)$$

the masses of end-groups of the four synthetic polymers were deduced from the MALDI spectra. In addition, combined with the information given by the commercial supplier, the structures of the end-groups of the four analytes were determined (shown in Table 3).

Quantitative Analysis

Three sets of experiments are included in the investigation. DI was used as the matrix. If no special cation salts are added, the PEGs are ready to form both sodium $[M + \text{Na}]^+$ and potassium $[M + \text{K}]^+$ adducts for the high affinity of PEGs with alkali salts and the trace presence of sodium and potassium coming from the solvent, matrix and so forth. We decided to apply the NaCl to analytes as the cationization salt for two reasons. First, the masses of $[\text{PEG} + \text{K}]^+$ and $[\text{P2} + \text{Na}]^+$ adducts are so close that their signal in the spectra

Table 3. The end-group information of the synthetic polymers

Polymers	Masses	Structure
M-PEG	15+17	$\text{CH}_3 + \text{OH}$
P1	69+31	$\text{H}_2\text{C}=\overset{\text{CH}_3\text{O}}{\underset{\text{O}}{\text{C}}}-\text{C}-\text{OCH}_3$
P2, P3	253+17	$n\text{-C}_{18}\text{H}_{37} + \text{OH}$

will be overlapped, e.g., the mass of 18-mer of $[\text{PEG} + \text{K}]^+$ is 863 Da while the mass of 13-mer of $[\text{P2} + \text{Na}]^+$ is 865 Da, which will directly obscure the calculation of integrated intensities. Second, the clear MALDI spectra with dominant sodium adduct signals are convenient for study.

The calculation of integrated signal intensities is based on the following formula:

$$I_t = \sum I_p \times \frac{M_p - M_{E1} - M_{E2} - M_C}{M_R} \quad (2)$$

where I_t represents the total signal intensity, I_p represents the intensity of each oligomer peak, M_p , M_{E1} , M_{E2} , M_R , and M_C represent the mass of each oligomer peak, the mass of two sides of end-groups, the mass of repeat unit, and the mass of cation, respectively. The ratios of the intensities of each component represent the quantity relationship of the components.

However, the biggest problem hindering the integrated intensity is sample inhomogeneity [21], which has long been the significant flaw of the MALDI method. A number of publications have represented the developed methods of decreasing the sample inhomogeneity, e.g., using liquid matrices [22], adding co-matrices to form a binary matrix [23], fast evaporation methods with fast evaporating solvents [24], electro-spray-based sample dotting method [25], and the sol-gel-based system [26]. All efforts contribute to the good co-crystallization of the sample. Herein, we started improving homogeneity from the sample mixing process. The mixed solutions of analyte, matrix, and cation salt were put into a supersonic wave chamber to make the mixture well-mingled. The homogeneity of the solution facilitates the homogeneity of the sample droplet deposited on the target using the air-dried method.

Another solution to reduce the sample inhomogeneity in our study is to introduce an internal standard. The inhomogeneous distribution of the sample spots would not be eliminated completely although the sample droplets have been well-treated, which is caused by factors like different evaporation time of solvent on the different positions of target and the differences in the solubilities of matrix and analytes in the solvent. Sample inhomogeneity decreases the repeatability of sample-to-sample and spot-to-spot measurements. The use of an internal standard can diminish the efforts of this serious flaw of inhomogeneity. In this paper, we chose **M-PEG** as an internal standard for its similar molecular properties to the analytes. The same repeat unit of $[\text{C}_2\text{H}_4\text{O}]$ may ensure the resemblance of the co-crystallization behavior when the internal standard mingled with the analytes.

Finally, the integral measurements have been carried out to compensate for the error resulting from the inhomogeneous distribution of the analytes within the matrix on the target. The laser has been randomly moved over the target spot at five points, where the spectra were obtained by accumulating data of

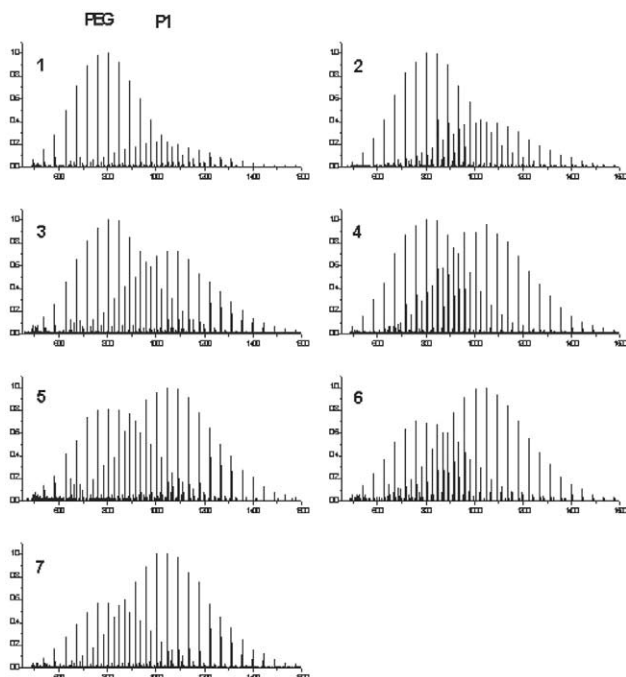


Figure 1. Stacked MALDI spectra of seven samples in Set A.

150 shots. Then the integrated intensities of the five spectra were calculated by eq 2 and averaged.

Set A

Standard **M-PEG** and analyte **P1** were mixed as the ratios listed in Table 1. The quantity of **M-PEG** was constant while that of **P1** varied from one volume to seven volumes. The stacked MALDI spectra under reflector mode are shown in Figure 1. Obviously, the intensities of **P1** increased from Figure 1, Sample 1 to Sample 7, and those of **M-PEG** little changed. The integrated intensities of **M-PEG** and **P1** in each spectrum were obtained through eq 2. As the internal standard, the integrated intensity of **M-PEG** acted as the divisor while that of **P1** acted as the dividend. The quotients of the division of five points of each sample were averaged, which is the basic data of our quantitative analysis (listed in Table 4).

As the numbers of moles of the analyte increased by

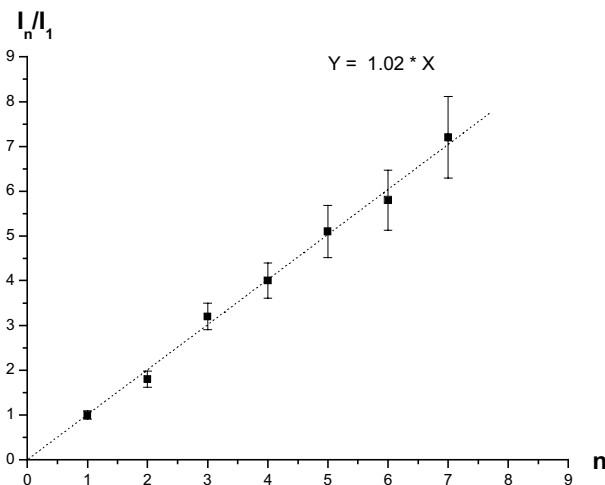


Figure 2. Plot of relative signal intensity ratios versus the quantity used ratios of **P1**.

degrees from Sample 1 to Sample 7, the relative integrated intensity ratios of the analyte and the standard (I_n) increased correspondingly. If the value of I_1 is regarded as a cardinal number with one volume of the analyte used in Sample 1, the value of I_n/I_1 is expected to represent number of volumes of the analyte use in Sample n . The results in Table 4 show that the integrated intensities ratios (I_n/I_1) exhibited good agreement with the ratios of the quantity of analyte used. A plot of relative integrated intensities (I_{P1}/I_{M-PEG} , as y axis) as a function of the volumes of **P1** used (as x axis) is drawn in Figure 2. The line passes through the origin by using a forced-fit, with a slope of 1.02 and correlation coefficient (R^2) of 0.997. In fact, the relationship between the normalized intensity ratio and the number of microliters of analyte implies the relationship between the measured intensity and the number of moles of analyte. The good linearity illustrates that the quantitative measurement of synthetic polymers with internal standard using MALDI-TOF MS is practicable.

Set B

In this set, we selected another synthetic polymer with different end-group composition from that of Set A as a

Table 4. Measured integrated intensities ratios of sets A–C

Set A			Set B			Set C		
Sample	$I_n (I_{P1n}/I_{M-PEG})^a$	I_n/I_1	Sample	$I_n (I_{P2n}/I_{M-PEG})$	I_n/I_1	Sample	$I_n (I_{P3n}/I_{M-PEG})$	I_n/I_1
A1	0.33 ± 0.03	1	B1	0.44 ± 0.04	1	C1	0.66 ± 0.08	1
A2	0.59 ± 0.06	1.8	B2	0.93 ± 0.09	2.1	C2	1.40 ± 0.12	2.1
A3	1.06 ± 0.10	3.2	B3	1.33 ± 0.15	3.0	C3	2.11 ± 0.22	3.2
A4	1.33 ± 0.13	4.0	B4	1.80 ± 0.19	4.1	C4	2.59 ± 0.28	3.9
A5	1.69 ± 0.19	5.1	B5	2.18 ± 0.23	4.9	C5	3.39 ± 0.35	5.1
A6	1.90 ± 0.22	5.8	B6	2.70 ± 0.30	6.1	C6	4.03 ± 0.42	6.1
A7	2.38 ± 0.30	7.2	B7	3.11 ± 0.35	7.1	C7	4.62 ± 0.49	7.0

^a I_n represents the ratio of I_P/I_{M-PEG} of the n th sample.

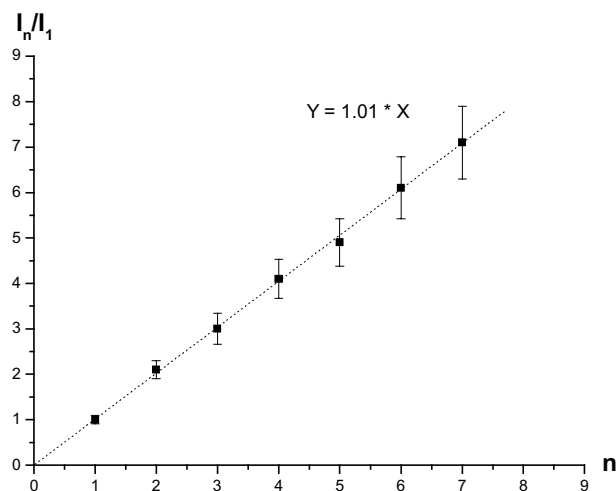


Figure 3. Plot of relative signal intensity ratios versus the quantity used ratios of **P2**.

supplementary proof to further test the practical utility of quantitative measurement on synthetic polymers. On the reflectron MALDI spectra, visible variation in intensities with the varied quantity of **P2** in each sample can be found. The results of integrated intensities calculation are listed in Table 4. Good agreement is found between the measured ratios and the anticipated one of stoichiometry. A plot with the measured relative ratios (I_n/I_1) as y axis and ratios of stoichiometry (n) as x axis is shown in Figure 3. The good linearity is exhibited on the slope of 1.01 and correlation coefficient (R^2) of 0.999, which supports the practicality of quantitative analysis of synthetic polymers on MALDI.

By analysis the data listed in Table 4 of Set A and Set B, we found that the relative intensity ratio of I_n of **P1** are different from that of **P2** with the same moles used; that is to say, the ionization efficiencies of **P1** and **P2** are different from each other. The ionization efficiency of **P2** is higher than **P1**. **P1** and **P2** are different in polymer properties. We think the difference is caused by the different end-groups of **P1** and **P2**, which have the same monomer and similar polymerization length. In the previous studies, PEGs are supposed to be ionized with alkali cation by wrapping around the cation [27]. In the case of **P1** and **P2**, we speculate that the large and hard acrylate end-groups of **P1** make the wrap more difficult than the soft and foldable long-chain-alkane of **P2**. The poorer wrap efficiency results in the lower ionization efficiency. A more systemic study will be carried on later. The similar results of end-group influence on ionization efficiencies on polystyrenes have been reported by Johnston and coworkers [28].

Set C

In the experiments of Set A and Set B, the three analytes are close in molecular weight distribution so the problem of low and high range mass discrimination of MALDI can not be represented. It is acknowledged that

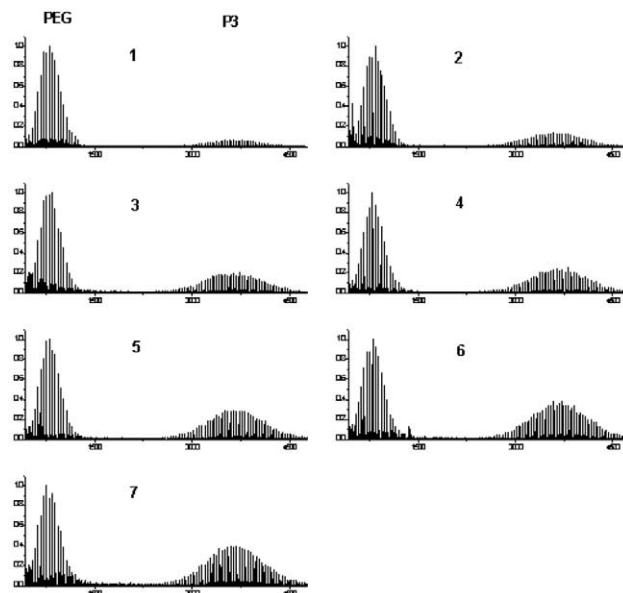


Figure 4. Stacked MALDI spectra of seven samples in set C.

the mass discrimination in the MALDI analysis does affect the signal intensity in the spectra [21]. To study the effect of mass discrimination on quantitative analysis, long length synthetic polymer **P3** has been applied to the investigation. **P3** is a kind of 80-mer PEGs with molecular weight average around 4 kDa compared to the 16-mer PEG standard with M_w around 800 Da. The stacked MALDI spectra under linear mode are shown in Figure 4. The detailed results of ratios of integrated intensities are shown in Table 4. The ratio results agree with the theoretical ratios calculated from stoichiometry. A plot was drawn according to the experimental data (see Figure 5). The good linearity of the plot (slope of 1.02, correlation coefficient $\{R^2\}$ of 0.998) proves the good quantitiveness disregarding the influence of mass discrimination. The ion intensity of **P3** is relatively low in the spectra compared to those of **P1** and **P2**. **P3**

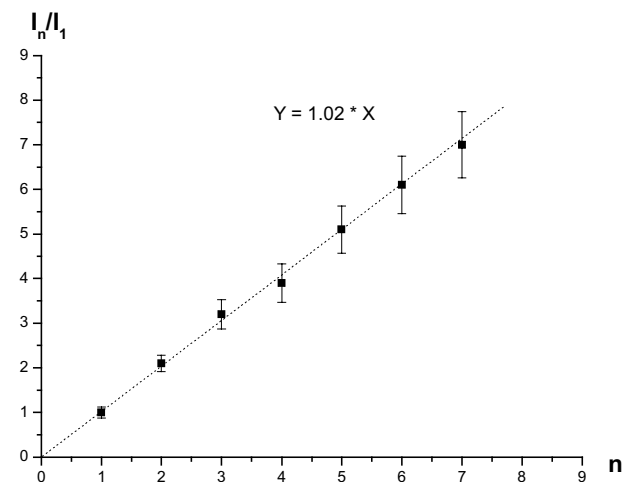


Figure 5. Plot of relative signal intensity ratios versus the quantity used ratios of **P3**.

has a lower intensity because the concentration of each individual oligomer is lower than that for **P1** and **P2** attributable to the broader molecular weight distribution. Another reason for the relatively low ion intensity of **P3** may be the detection efficiency. Channel plate detectors of MALDI-TOF MS have a limited dynamic range and can get saturated easily by low molecular weight components. Only when more abundant low mass ions of the M-PEG are deflected from reaching the detector, can high mass ions of **P3** be observed.

Conclusions

MALDI-TOF mass spectrometry has been used to conduct quantitative analysis on synthetic polymers. Several actions have been taken to optimize quantitative performance, e.g., the supersonic wave treat of sample mixture, the use of an internal standard and the average of randomly selected spots on the target. This is the first report using an internal standard for quantitative analysis of synthetic polymers by MALDI-TOF. The results of the three sets, A–C, show us good linearity with nice agreement on the observed integrated intensity ratios and the stoichiometry ratios, which demonstrate that the quantitative measurements in our study, as well as the qualitative characterization of the synthetic polymers, were a success. The utilization of an internal standard provides us good results due to the advantages of improving the sample-to-sample and spot-to-spot repeatability caused by the sample inhomogeneity, ignoring the difference of ionization efficiencies among the components in polymers and limited influence by the low and high range mass discrimination within the MALDI performance. Furthermore, the effects of end-groups on the ionization efficiency of polymers and the detection differences are observed. Both the practical quantitative measurement of different components of the same polymer in the previous study [20] and that of a fixed component in different polymers in present study prove the practicability of quantitative analysis on synthetic polymers using MALDI-TOF MS.

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