Selection of Internal Standards for Quantitative Matrix-Assisted Laser Desorption/Ionization Mass Spectrometric Analysis Based on Correlation Coefficients

Shumei Yang,† Lei Mu,† Ruxia Feng,† and Xianglei Kong‡,*†

†The State Key Laboratory of Elemento-Organic Chemistry, College of Chemistry and ‡Collaborative Innovation Center of Chemical Science and Engineering, Nankai University, Tianjin 300071, P. R. China.

ABSTRACT: Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) has shown its great success in the qualitative analysis of a wide range of organic and biological molecules. However, its application in quantitative analysis is still limited by the difficulty in the availability of isotope-labeled internal standards. The present work investigates the relationship between the correlation coefficient of the peak intensities of analyte and candidate internal standard ions and the linearity of possible quantitative analysis. Based on the two analyte examples, ciprofloxacin and substance P, the results show that the performance of the selected nonisotope-labeled internal standard is greatly related to the correlation coefficient. A high positive correlation coefficient (>0.7) between the ions of analyte and candidate standard can result in a good linearity (R² > 0.98) and vice versa. The results provide a new way to select nonisotope-labeled internal standards for MALDI analysis and thus can be potentially applied in the rapid quantitative mass spectrometry.

INTRODUCTION

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) has been applied for the analysis of a wide range of molecules since its inception in the late 1980s.1,2 Over the years, MALDI MS has been an indispensable tool in related research fields in chemistry and biology.3–5 Despite its good performance, MALDI MS still has a significant limitation in quantitative analysis.6–9 Generally, there are two ways to solve this problem: modifying the general MALDI approach or choosing suitable matrices to generate a uniform distribution of analytes and matrices and applying internal standards in MALDI analysis. For the first one, several groups developed different ways to enhance the homogeneity of the target surface.10–14 For example, Thomas et al. reported that desorption/ionization on silicon (DIOS) allows for the analysis of related small molecules in the absence of matrix with a good reproducibility.7 Nie et al. used carbon nanodots (CDs) with excellent water solubility as the matrix for quantified MALDI-TOF MS analysis of glucose in serum.12 Gross and colleagues have reported the use of ionic matrices in quantitative applications of oligodeoxynucleotides, peptides, and small proteins, which have good calibration linearity and reproducibility.10,13,14 Oh et al. have reported the use of the ionic liquid matrix, 1-Melm-CHCA, which has a good reproducibility to quantify polyhexamethylene guanidine and obtained a good linear calibration.15

Practically, the second way is more widely used than the first one. Internal standards are incorporated into quantitative mass spectrometric analyses to compensate for both systematic and random errors during analyses and provide reliable results.16–18 It is very important to choose an appropriate molecule as the internal standard for the special analyte. A good internal standard for quantitative analysis should have physical and chemical properties similar to those of the analyte of interest and should have an ionization efficiency comparable to that of the analyte. Surely, an ideal internal standard is a stable isotope-labeled form of the analyte. However, in most of the time, the isotope-labeled standards are costly or commercially unavailable or even just unavailable due to their great difficulty in synthesis. Hence, the exploration of how to choose a nonisotope-labeled internal standard with stable properties and low cost is urgent.

Thus, the key is whether we can set up a method to find these nonisotope-labeled internal standards. Sleno and Volmer have noticed the point and found that the distribution coefficient, log D, could function as a fundamental parameter for similar crystallization patterns of analyte and selection of internal standards.20 Here, we devoted ourselves to fulfill the needs using a different way with the aid of correlation analysis. Correlation analysis has been applied for studying the mechanism of ion formation in MALDI and other relative topics by several groups.23–29 For example, Feldman et al. used...
the event-by-event analysis method to study sample surface heterogeneity and elucidate the mechanisms of ion formation in MALDI-TOF.27 Using this method, we have discovered the competition between metal cationization and protonation/reduction in MALDI process of riboflavin recently.28 Briefly, the peak intensity variations of signals can reflect the correlation relationship between the ions. In the MALDI process, a positive correlation coefficient means the intensities of the two ions increase or decrease together, indicating that they have similar formation processes. In this study, we analyze the relationship between the correlation coefficient and linearity of quantitative analysis. The interesting results show how the correlation coefficient can assist us to choose appropriate nonisotope-labeled internal standards effectively.

Results and Discussion. In order to minimize the effects on peak intensity caused by many unknown factors in the MALDI process, the signal reproducibility is extremely important. Thus, besides the widely used matrix of α-cyanoguanine-4-hydroxycinnamic acid (CHCA), the ionic liquid matrix of 1-methylimidazolium-cyan-4-hydroxycinnamate (1-MeIm-CHCA) was also selected here. The latter matrix reported by Yoon et al. has shown good reproducibility and been applied in the quantitative analysis of polyhexamethylene guanidine (PHMG).15 To examine the signal reproducibility, the samples of ciprofloxacin were prepared with both matrices of CHCA and 1-MeIm-CHCA. For each of them, we collected 50 successive mass spectra at different locations in the same spot by continuous moving the target in a zig-zag way. The intensities of the protonated ions ([Cip + H]+) are summarized in Figure 1. Comparing with the poor reproducibility in the case of CHCA matrix, the matrix of 1-MeIm-CHCA does show good reproducibility. If other metal-cationized ions (such as [Cip + Na]+) were compared, the results are similar. This is mainly due to the more homogeneous sample surface generated by 1-MeIm-CHCA, which forms a transparent liquid film on the sample. The spot-to-spot reproducibility was also evaluated according to the five spots prepared in the same metal target, and the standard deviation of the intensities of [Cip + H]+ is found to be less than 5% for the matrix of 1-MeIm-CHCA. Briefly, the matrix of 1-MeIm-CHCA has a homogeneous surface and can greatly reduce the sample-to-sample and spot-to-spot variabilities and thus been applied in this research to explore the relationship between the quantitative analysis and correlation analysis.

Ciprofloxacin (MW = 331.2) is selected as the first sample to be analyzed here, and six compounds are chosen as candidates for internal standards. All their structures are shown in Scheme 1. In the present study, the MALDI-TOF mass spectra were acquired for ciprofloxacin prepared at different concentrations (from 5 μM to 1.5 mM), with D8-ciprofloxacin, sarafloxacin, enrofloxacin, enalapril, 3,5-diiodo-L-tyrosine, and quinidine added at a fixed concentration as internal standards. Figure 2 shows the representative mass spectra of ciprofloxacin obtained under four different concentrations, with sarafloxacin as the internal standard. Signals from the analyte, including [Cip + H]+, [Cip + Na]+, [Cip + K]+, and those from internal standard, were observed. When the ciprofloxacin concentration was less than 5 μM, no ciprofloxacin signal was detected. When its concentration was higher than 1.5 mM, the internal standard signal was too weak. Therefore, the concentration range of ciprofloxacin was selected from 5 μM to 1.5 mM when the internal standard was fixed at 0.3 mM in all these experiments.

In some cases, the metal-cationized signals of internal standards cannot be observed in the mass spectra. Thus, protonated ions of [Cip + H]+ and the internal standard ([IS + H]+) were applied here for the quantitative analysis. Calibration plots based on logI resolved (I∗/Iσ0) versus logI analyte are shown in Figure 3, in which the peak intensities of the analyte and internal standard observed in mass spectra are indentified as I∗ and Iσ0 and the concentration of the analyte in the solution is r indenitified as [analyte]. Figure 3a shows the calibration curve obtained with the internal standard of D8-ciprofloxacin. As expected, the standard isotope-labeled compound does behave as an ideal internal standard. A very good linearity was obtained, and the linear correlation coefficient (R2) was reported to be 0.9985. The two peaks of [Cip + H]+ and [D8-Cip + H]+ are found to be greatly positively correlated, with all correlation coefficients between I∗ and Iσ0 obtained under nine concentrations satisfied r > 0.93 (Figure 3b).

When sarafloxacin was applied as the internal standard, the linearity decreased to 0.9871. And it is also found that the ions of [Cip + H]+ and [Sar + H]+ are greatly positively correlated, with all other correlation coefficients satisfied r > 0.90, except the ones at the three lowest concentrations (Figure 1b,c). Enrofloxacin gives similar results. As shown in Figure 1d,e, the concentration coefficients of enrofloxacin and ciprofloxacin were found to be larger than 0.8, except for the cases with the lowest and highest concentrations, and the linearity is 0.9875. While for enalapril, the average correlation coefficient decreases and the linearity also decreases to 0.9777 (Figure 3g,h). As a negative example, 3,5-diiodo-L-tyrosine was also selected as a failed internal standard for ciprofloxacin due to its different physical and chemical properties. Not surprisingly, the linearity was just R2 = 0.8658. Interestingly, the correlation coefficients obtained under different concentrations have negative values from −0.3 to −0.8. High values of standard deviations were also observed.

As a different example, quinidine does not belong to the group of 4-quinolones. The compound is an antibiotic with a different structure from ciprofloxacin. Interestingly, Sleno and Volmer have found that it can be used as a good internal standard for quantitative MALDI analysis of ciprofloxacin (with a linearity of 0.99).22 Here, quinidine was also tested. The results are shown in Figure 3i. It is also found that quinidine and ciprofloxacin have a very strong positive correlation. Except for the lowest concentration example, the correlation coefficients are all above 0.85. Correspondingly, it is believed that quinidine can be applied as a good internal standard...
standard, and it is proved by the experimental results shown in Figure 3k, in which the linearity was $R^2 = 0.9954$.

In order to further prove the relationship between the correlation coefficient and linearity, substance P, an undecapeptide, was selected as the second example. Three different kinds of compounds: angiotensin II, $\beta$-CD, and C$_{60}$ were chosen as internal standards. Among them, angiotensin II is also a peptide and the latter two are totally different compounds with very different chemical compositions and properties, which are purposely selected here as negative examples. Calibration curves and correlation results are presented in Figure 4. The concentrations of substance P were prepared from 1 $\mu$M to 425 $\mu$M in these experiments. Not surprisingly, the results show that angiotensin II has the best linearity with $R^2 = 0.9944$ and good correlation coefficients with all values of $r$ larger than 0.85 except for the experiment with the lowest concentration of substance P at 1 $\mu$M (Figure 5a,b). When $\beta$-CD was applied as the internal standard, the linearity decreased to 0.9615, and the correlation coefficients also became weak. As another example with poor correlation coefficients, fullerene of C$_{60}$ was also tested here. The results show weak negative correlation coefficients and very poor linearity. It also indicates that if the compound and analyte have a weak correlation, the compound is not suitable for acting as an internal standard.

In order to make sure the performability of the method in the quantitative mass spectrometry analysis for realistic samples, urine samples were also tested here. Standard solutions of ciprofloxacin with different concentrations were prepared by dissolving the compound in fresh human urine samples, and sarafloxacin was selected as the internal standard here. Both ciprofloxacin and sarafloxacin can be directly detected in the forms of $[\text{Cip} + H]^+$, $[\text{Cip} + \text{Na}]^+$, $[\text{Cip} + K]^+$, $[\text{Sar} + H]^+$, $[\text{Sar} + \text{Na}]^+$, and $[\text{Sar} + K]^+$. It has been found that all the protonated pairs and metal-cationized pairs could be applied for the quantitative analysis. As shown in Figure 4a, the calibration curve was plotted according to logarithmic values of ratios of $I_{[\text{Sar} + \text{Na}]^+}/I_{[\text{Cip} + \text{Na}]^+}$. A good linearity with $R^2 = 0.9885$ in the concentration range of 0.01–1.5 mM was observed, very close to the results shown in Figure 2c,d. Correspondingly, except for the two cases with the lowest concentrations, all other correlation coefficients are larger than 0.8 (Figure 5b). For the case of substance P in the urine sample with the angiotensin II as the internal standard, similar results were observed (Figure 5c,d). These experimental results do reflect the potential of the method in real sample analysis. However, further systematic studies about its performability are still needed very much.

Through the analyses of correlation coefficients and linearity shown above, it is believed that the performance of a candidate internal standard is greatly related to the correlation coefficients between it and the analyte reflected in the MALDI experiments. The higher the correlation coefficient, the better linearity can be achieved. If the correlation coefficient is below 0.7, the compound is suggested to be not suitable as an internal standard of the analyte. Practically, the method can be used to screen the best internal standards from some candidates with kinds of structural similarities to the analyte. It starts from the analysis of correlation coefficients among the candidates and analyte and then the candidates with high correlation coefficients with the analyte are preferentially selected for further linearity test.

### CONCLUSIONS

The present work investigates the selection of internal standards for quantitative analyses using the method of MALDI mass spectrometry. In order to discover the relationship between the correlation coefficient and quantitative analysis, spot-to-spot and shot-to-shot differences should be reduced. In order to perform this, the ionic liquid matrix of

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**Scheme 1. Compounds Used in the Study of Ciprofloxacin and Their Molecular Weights Are Also Shown**

| Compound        | Molecular Weight |
|-----------------|------------------|
| ciprofloxacin   | 331.35           |
| sarafloxacin    | 385.36           |
| enrofloxacin    | 359.40           |
| enalapril       | 376.45           |
| quinidine       | 324.42           |
| 3,5-diiodo-L-tyrosine | 432.98 |
1-MeIm-CHCA was applied here to obtain homogeneous sample spots. Two kinds of analytes (ciprofloxacin and substance P) were selected here. It is observed that the performance of the candidate internal standard is greatly related to the correlation coefficients between it and the analyte. When the corresponding ions of the candidate internal standard and the analyte of interest are highly positively correlated (>0.7), the candidate might be used as a good internal standard. The higher the correlation coefficient is, the better the linearity of the calibration curve and the less the standard deviation are. The results provide a new way to help us select internal standards for quantitative MALDI analysis and thus can be potentially applied in the field, especially in cases when the isotope-labeled standards are unavailable.

**METHODS**

**Experimental Section.** **Chemicals and Reagents.** D8-ciprofloxacin hydrochloride hydrate, angiotensin II (human), substance P acetate salt hydrate, CHCA, and 3,5-diiodo-L-tyrosine dihydrate were purchased from Sigma-Aldrich. Ciprofloxacin and enrofloxacin were obtained from Energy Chemical (Shanghai, China). Sarafl oxacin was purchased from J&K Scientific Co. Ltd. (Beijing, China). β-cyclodextrin was purchased from Aladdin (Shanghai, China). C_{60} was purchased from TCL (Shanghai, China).

**Sample Preparation.** The ionic liquid matrix of 1-MeIm-CHCA was prepared using the method previously described.15 Briefly, 1 mL of CHCA (0.2 mol/mL in MeOH) was thoroughly mixed with 0.15 mol of liquid 1-methylimidazole by vortexing and sonicating for 10 min. The mixture was then
allowed to react at room temperature for 10 min and dried under nitrogen. The dried, yellowish product was dissolved in MeOH at a concentration of 70 mg/mL, and this solution was used as an ionic liquid matrix.

Ciprofloxacin and substance P were selected as the target analytes in the experiments. Standard solutions of ciprofloxacin and substance P were prepared by directly dissolving the corresponding chemicals in water at concentrations of 3 mM and 0.85 mM as stock solution, respectively. For ciprofloxacin, the stock solution was then diluted to become solutions with different concentrations of 5, 10, 20, 40, 80, 150, 300, 600, and 1500 μM. Sarafloxacin enrofloxacin, enalapril, and quinidine were prepared with a concentration of 0.3 mM in water when they were used as internal standards, while D8-ciprofloxacin and 3,5-diiodo-L-tyrosine were prepared with concentrations of 0.1 and 10 mM, respectively. Solutions of substance P were prepared by gradient dilution to become solutions with different concentrations of 1, 5, 10, 40, 80, 100, 150, 250, and 425 μM. The concentrations of angiotensin II and β-cyclodextrin were both chosen to be 0.1 mM, and that of C60 was 3 mM.

The urine sample was collected from a healthy female volunteer. After a centrifugation at 16,000 rpm for 30 min, the top two-thirds of the urine was transferred into tubes. After that, 10 μL of the analyte was premixed with 10 μL of 1-MeIm-CHCA matrix in a centrifuge tube, and then, the mixture was diluted 20 times with water. Then 1 μL of the resulting solution was deposited on the target plate and air-dried for further MS analysis.

MALDI-TOF MS

Data were acquired using a Bruker Daltonics MALDI time-of-flight (TOF) mass spectrometer (Autoflex III) equipped with an Nd:YAG laser at a wavelength of 355 nm. The repetition rate of the laser is 20 Hz. The instrument was operated in a positive ion reflection mode under delayed extraction condition with a source potential of 17 kV. Each mass spectrum reported here was calibrated automatically using the charged ion species from the matrixes of CHCA by an external spot nearby before the start of each acquisition. Each spectrum was acquired by accumulating ion signals of 500 consecutive laser shots obtained on the same sample spot. Each correlation coefficient is calculated based on 20 mass spectra by continuous acquisition without any selection.

Covariance Mapping. The correlation coefficient is calculated by

$$r(x, y) = C(x, y) / \sqrt{C(x, x)C(y, y)}$$

in which C(x,y) is the covariance matrix, and it is calculated by

$$C(x, y) = \frac{1}{n} \sum_{i=1}^{n} (x_i - \bar{x})(y_i - \bar{y})$$

where x_i and y_i represent the ion signal intensities at the i-th point; \(\bar{x}\) and \(\bar{y}\) are the mean signal intensities at the same point for all measured mass spectra. The total number of experiments is indicated by n.

AUTHOR INFORMATION

Corresponding Author

*E-mail: kongxianglei@nankai.edu.cn. Phone: +89 22 23509564.
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Foundation of China (grant nos. 21627801 and 21475065).

This work was supported by the National Natural Science Foundation of China (grant nos. 21627801 and 21475065).

ACKNOWLEDGMENTS

The authors declare no competing financial interest.

**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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