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Qualitative and Quantitative Analysis of Small Amine Molecules by MALDI-TOF Mass Spectrometry through Charge Derivatization

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A pair of isotopically coded light and heavy reagents, tris-(2,4,6-trimethoxyphenyl)phosphonium acetic acid *N*-hydroxysuccinimide esters (1 and 2) were synthesized and used to derivatize low molecular weight (<500 Da) molecules containing primary or secondary amine functional groups for MALDI-TOF MS analysis. The light and heavy reagents added a 573 and 600 Da positively charged tag to each analyte, respectively. In the presence of 10 times excess of tag reagents, the coupling reactions reached near completion within 10 min. The derivatization greatly facilitated MALDI analysis of small molecules and significantly improved the sensitivity of analysis, allowing a limit of detection in the low femtomole range. Additionally, the reaction mixtures were directly analyzed by MALDI without sample cleanup. The quantification of small molecules by MALDI-TOF MS was successfully achieved by analysis of isotopically coded light and heavy derivatives. MALDI-TOF quantitative analysis of a mixture of antibiotics yielded calibration curves in the concentration range from 0.3 to 30 pmol/μL with r^2 values greater than 0.9995.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry has become a widely used and powerful tool for analysis of biomolecules and synthetic polymers.[1] Protein identifications via the peptide mass fingerprinting (PMF) approach employ MALDI-TOF to analyze protein digests.[2] MALDI has also been utilized for genotyping single nucleotide polymorphisms (SNP) generated from PCR reactions.[3] The wide application of MALDI-TOF MS has been attributed to some of the major advantages of the technique, such as the simplicity of usage, high sensitivity, reasonable tolerance against impurities, and the potential of high sample throughput. Despite the vast success of MALDI-TOF MS in the analysis of large molecules, difficulties may arise for the MALDI analysis of low molecular mass compounds (so-called small molecules, MW < 500 Da). There are several obstacles for successful application of MALDI-TOF MS to the analysis of small molecules. First, the analytes of interest may have very poor ionization efficiency due to the lack of high-proton-affinity functional groups. Second, the presence of a variety of abundant matrix-related ions in the low-mass range clutters the spectrum below 500 Da. Also, matrix ions could suppress analyte signals and may cause isobaric overlay with analyte peaks in the MALDI spectrum. To widen the applicability and to fully take advantage of the MALDI-TOF technique, several alternative approaches have been proposed to improve the measurability of low molecular weight analytes by MALDI-TOF MS.[4−8] Gou et al.[4] utilized a surfactant in an attempt to suppress the matrix signals and therefore differentiate them from the analyte signals. Siuzdak et al.[6] reported qualitative analysis results on small molecules using the desorption/ionization on silicon (DIOS) approach. Overall, qualitative and quantitative MALDI-TOF analysis of small molecules remains a challenge.

Our strategy to analyze small molecules via MALDI-TOF MS was to use a simple and efficient derivatization reaction to add a large positively charged tag to the analytes. Many potential advantages can be realized by derivatizating low-molecular weight molecules with a large charged tag for MALDI analysis. These include increased signal intensities due to improved ionization efficiency, a mass shift toward higher mass range to differentiate analyte ions from matrix ions and an unambiguous identification of a class of compounds in a mixture. Further, by incorporation of isotopes into the derivatizing reagents, reliable quantitative analysis of small molecules could be achieved.

In this work, we report our investigation on derivatizing small molecules with *N*-hydroxysuccinimide ester **1** and its heavy-isotope labeled analogue **2** for MALDI-TOF MS. Reagent **1** was first reported by Huang et. al. as a reagent for *N*-terminal peptide modification of tryptic digests.[9−13] For this study, a variety of

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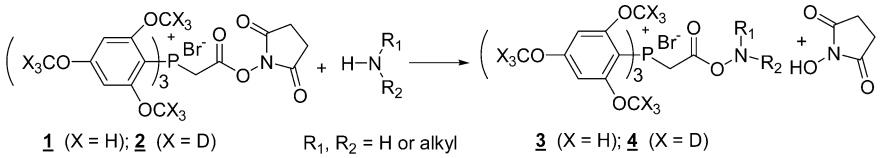
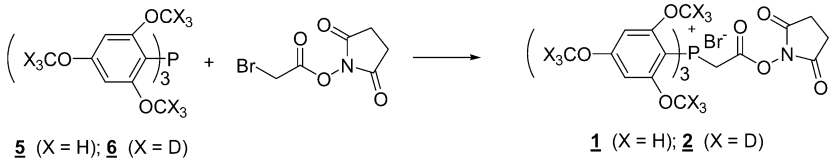
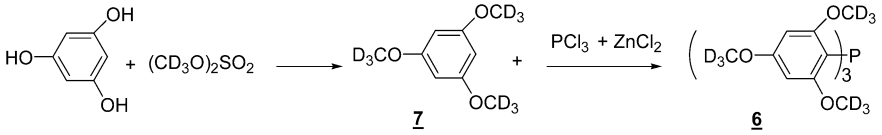
Scheme 1



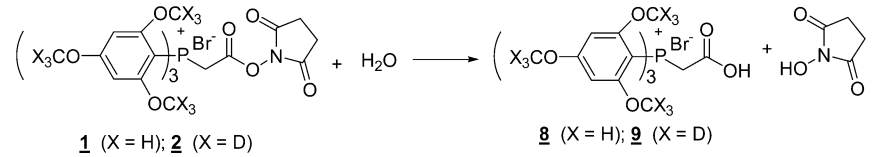
Scheme 2



Scheme 3



Scheme 4



primary and secondary amines including alkylamines, drugs, antibiotics, and stimulants were derivatized by **1** and **2**, adding 573 and 600 Da of a positively charged tag to each analyte (**3** and **4**, Scheme 1). The derivatization procedure was tested and optimized with respect to simplicity, efficiency, and measurability of the derivatives by MALDI-TOF MS. In addition, the quantitative analysis of small molecules by MALDI-TOF MS was performed.

EXPERIMENTAL SECTION

Reagents. Anhydrous acetonitrile (CH_3CN), cyclooctylamine, dimethyl-d_6 sulfate, dioctylamine, enoxacin, lomefloxacin, norfloxacin, naphthalenemethylamine, phosphorus trichloride, triethylamine, triethanolamine, trihydroxybenzene, triethylaminonium bicarbonate buffer (1.0 M), tris(2,4,6-trimethoxyphenyl)-phosphine (**5**), and zinc chloride undecylamine were purchased from Sigma-Aldrich (St. Louis, MO). Phentermine was purchased from Alltech (Deerfield, IL) and ciprofloxacin was purchased from Serologicals (Canada). Trifluoroacetic acid (TFA) was purchased from Fisher Scientific (Pittsburgh, PA) and ultrapure water (18.2 MΩ) was used. α-Cyano-4-hydroxycinnamic acid was obtained from Waters (MassPREP MALDI Matrix CHCA). Compounds **1** and **2** were prepared by reacting **5** or tris(2,4,6-trimethoxy-d_9)-phenyl)phosphine (**6**) with bromoacetic acid *N*-hydroxysuccinimide ester, respectively, in toluene for 30 min using published procedures (Scheme 2).[7,9] Compound **6** for preparing **2** was synthesized using published methods (Scheme 3).[14,15] Basically, trihydroxybenzene, dimethyl-d_6 sulfate, and potassium carbonate were refluxed in acetone for 3 h to yield **7**.[14] The isolated **7** was mixed with $ZnCl_2$ and refluxed in phosphorous trichloride solution for 8 h to yield **6**.[15]

MALDI-TOF MS Analysis. Spectra were acquired with a Micromass M@LDI LR time-of-flight mass spectrometer (Waters Corparation, Milford, MA) using a 337-nm nitrogen laser and an accelerating voltage of 15 kV. The instrument has delayed extraction capabilities, and the delay time setting was 500 ns. All the data were acquired using positive reflectron mode, and each spectrum represented a sum of 100 laser shots. The ion extraction pulse voltage was fixed at 2000 V. Data collection and processing were controlled by the manufacture's software.

High-purity CHCA was used as the MALDI matrix throughout this study. It was dissolved in 50% acetonitrile and 50% ethanol at a concentration of 10 mg/mL. Samples for mass spectrometry were prepared by mixing the acidified reaction mixture with the matrix solution in a 1:1 ratio (v/v) and spotting 1 μL of this onto a stainless steel target plate. The applied samples were allowed to dry at ambient temperature before transferring into the mass spectrometer.

Preparation of Stock Solutions. Stock solutions of **1** and **2** (12 nmol/μL) were prepared by placing 13.1 mg of **1** and 13.7 mg of **2** into 1400 and 1415 μL of anhydrous acetonitrile, respectively, in a 1.5-mL screw cap polypropylene microcentrifuge tube. *N*-hydroxysuccinimide esters **1** and **2** were reacted with water to form carboxylic acids **8** and **9** when they were exposed to water (Scheme 4). At ambient temperature, the half-life time of **1** in unbuffered CH_3CN/H_2O (50/50 v/v) solution is 18 h and the half-life in pH 9.2 buffer solution is 40 s. Care must be taken to avoid exposing the stock solution to moisture or buffer solutions until required. The stock solutions can be stored in a desiccator at room temperature for 3 months without discernible decomposition as determined by ESI-MS or MALDI-MS.

Three buffer solutions (16 mM triethylamine, triethanolamine, and triethylaminonium bicarbonate), each containing 20% (v/v)

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of CH_3CN, were used in this study. Buffer A (pH 11.7) was prepared by mixing 0.810 g of triethylamine with 400 mL of water and 100 mL of acetonitrile. Buffer B (pH 9.9) was prepared by mixing 1.194 g of triethanolamine with 400 mL of water and 100 mL of acetonitrile. Buffer C (pH 9.2) was prepared by by mixing 8 mL of triethylaminonium bicarbonate (1.0 M) solution with 392 mL of water and 100 mL of acetonitrile.

Derivatization of Amines. The method described below is a generic procedure used for derivatization of amines. Analytes were dissolved in the buffer solutions (A, B, or C). To 400 μL of analyte solution (4 pmol/μL) was added 8 μL of **1** or **2** (12 nmol/μL) stock solution. The solution was mixed with a vortex mixer for 10 s and incubated at room temperature for 10 min. At the end of the reaction, the reaction mixture was acidified with TFA to pH 2.0.

Comparison of Coupling Yield at Different pH. The amines **10**−**15** (Figure 1) were placed in buffer A, B, and C to make 4 pmol/μL of analyte solution D, E, and F, respectively. Analyte solutions D and E were derivatized with **1**, and analyte solution F was derivatized with **2**, using the derivatization method described to yield solutions D-**1**, E-**1**, and F-**2**, respectively. Equal volumes of solutions D-**1** and F-**2** were mixed to make sample I and equal volumes of solutions E-**1** and F-**2** were mixed to make sample II. Samples I and II were acidified with TFA and analyzed by MALDI-TOF MS. The peak intensity of derivatives in D-**1** and F-**2** of sample I and derivatives in E-**1** and F-**2** of sample II were recorded in Tables 1 and 2, respectively. The peak intensity of derivatives in D-**1**, E-**1**, and F-**2** in the spectra of samples I and II were further normalized using the following equations ((1)−(3)):

$$J = \frac{(n + m)}{2} \tag{1}$$

$$K = \frac{w(n + m)}{2n} \tag{2}$$

$$L = \frac{z(n + m)}{2m} \tag{3}$$

where J is the normalized peak intensity of derivatives in F-**2**, K is the normalized peak intensity of derivatives in D-**1**, L is the normalized peak intensity of derivatives in E-**1**, n is the peak intensity of derivatives in F-**2** in the sample I spectrum, m is the peak intensity of derivatives in F-**2** in the sample II spectrum, w is the peak intensity of derivatives in D-**1** in the sample I spectrum, and z is the peak intensity of derivatives in E-**1** in the sample II spectrum.

Stoichiometry. Antibiotics **16**, **17**, and **18** were dissolved in buffer C to prepare 9 pmol/μL of antibiotic stock solution. To six microcentrifuge tubes containing 1 mL of the antibiotic solution was added 10, 20, 30, 40, 50, and 100 μL of **1** (12 nmol/μL), respectively. Each solution was mixed with a vortex mixer for 10 s and incubated at ambient temperature for 10 min to yield derivatives of **1**. To those microcentrifuge tubes, 50 μL of **2** (12 nmol/μL) was added and incubated for 10 min to convert any unreacted antibiotic to derivatives of **2**. Then, each solution was acidified with TFA and added matrix and subjected to MALDI-TOF analysis. The peak intensities of the derivatives of **1** and their corresponding derivatives of **2** on each spectrum were examined and used to calculate the percent yield of the coupling reaction of **1** of those six solutions as shown in eq 4.

$$\text{yield}(\%) = \frac{100x}{(x + y)} \tag{4}$$

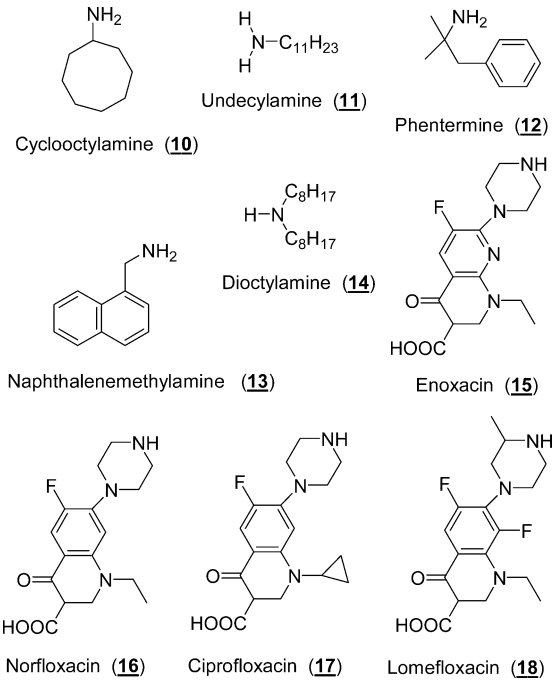

Figure 1. Structure of analytes.

Table 1. Comparison of pH 11.7 and 9.2 for Coupling Reaction

	peak intensity	
analytes	derivative of **1** (pH 11.7)	derivative of **2** (pH 9.2)
primary amine		
10	8374	712
11	3278	1390
12	3924	1013
13	10326	13990
secondary amine		
14	251	1909
15	5020	8342

Table 2. Comparison of pH 9.9 and 9.2 for Coupling Reaction

	peak intensity	
analytes	derivative of **1** (pH 9.9)	derivative of **2** (pH 9.2)
primary amine		
10	1943	647
11	2962	1516
12	9752	987
13	16308	14475
secondary amine		
14	3520	3349
15	6313	6442

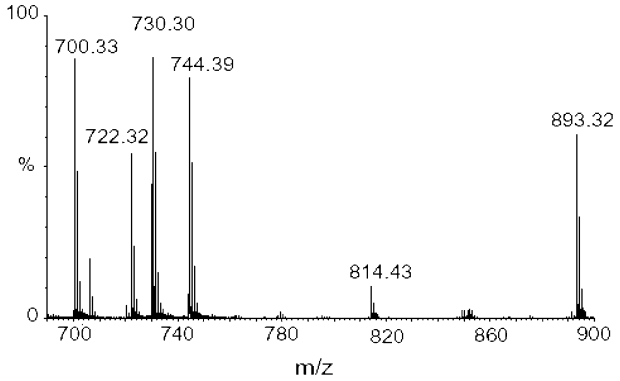

Figure 2. MALDI-TOF spectrum of 2 pmol of derivatized **10** (C^+ = 700.33), **12** (C^+ = 722.32), **13** (C^+ = 730.30), **11** (C^+ = 744.39), **14** (C^+ = 814.43), and **15** (C^+ = 893.32).

where x is the peak intensity of the derivative of **1** and y is the peak intensity of its corresponding derivative of **2**.

RESULTS AND DISCUSSIONS

A mixture of six amines, **10**−**15** (4 pmol/μL), was derivatized by **1** in buffer A (pH 11.7). The selected compounds represent some primary and secondary amines with a selection of aliphatic and aromatic moieties coupled to various combinations of chemical functional groups as shown in Figure 1. The *N*-hydroxysuccinimide ester of **1** was reacted with an amine functional group to form a stable amide linkage, adding 573 Da to each analyte. Figure 2 shows the MALDI-TOF spectrum of the resulting derivatives. The peak intensities of the derivatized **10**, **11**, and **13** ions were among the strongest, followed by the derivatized **12** and **15** ions. The peak intensity of the derivatized **14** ion was the lowest among the six derivatives, which is possibly a result of the lower conversion yield of the coupling reaction due to the steric hindrance of the two long alkyl chains surrounding the amine group, or from poor cocrystallization with the matrix (CHCA).

The competitive reactions of amidation (Scheme 1) and hydrolysis of the *N*-hydroxysuccinimide esters **1** and **2** (Scheme 4) occur spontaneously during derivatization in aqueous solution. The basic buffer solutions were used to facilitate the condensation reaction, and a large excess of **1** was frequently added to ensure high yields of derivatization of the amine analytes.[9] Several buffer systems have previously been reported for the charge derivatization reaction,[9−11] including phosphate, Tris−HCl, BICINE, and HEPES. The use of these buffers requires sample purification prior to mass analysis since they are not volatile. Also, buffers such as Tris−HCl can compete with analytes to react with **1** and **2** during derivatization. Because MALDI-TOF was chosen as the analytical technique for the analysis of derivatized compounds in this study, it was desirable to choose votatile buffers that can provide buffer capacity in the desired pH range and also minimize interferences during MALDI analysis.

Since the rate of reaction is likely to be analyte-specific, optimizing the pH of buffers and the stoichiometry will increase the yield of derivatization and decrease the amount of hydrolyzed *N*-hydroxysuccinimide ester in the reaction solution. HPLC analysis was used to analyze the yield of *N*-hydroxysuccinimide ester **1** derivatized analytes. However, it was a time-consuming process for obtaining suitable internal standards and optimizing separation conditions.[9,12] A fast and simple MALDI-TOF MS method, which analyzes the relative amount of isotopically coded light **1** and heavy **2** derivatives, was developed to compare and optimize the coupling reaction conditions.

Table 3. Comparison of pH 11.7, 9.9, and 9.2 for Coupling Reaction

	normalized peak intensity		
analytes	derivative of **1** (pH 11.7)	derivative of **1** (pH 9.9)	derivative of **2** (pH 9.2)
primary amine			
10	7992	2040	679
11	3425	2839	1453
12	3872	9883	1000
13	10504	16035	14232
secondary amine			
14	345	2763	2629
15	4448	7245	7392

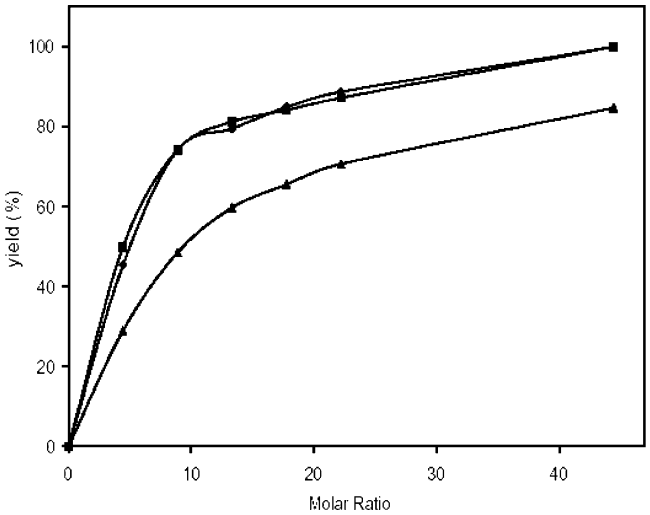

Figure 3. Plots of the percent conversion yield versus the molar ratio of **1** to antibiotics **16** (◆), **17** (■), and **18** (▲).

Optimization of pH for Coupling Yield. Three volatile buffers, A (pH 11.7, 16 mM triethylamine), B (pH 9.9, 16 mM triethanolamine), and C (pH 9.2, 16 mM triethylaminonium bicarbonate), were selected to provide a pH range for investigating the efficiency of coupling **1** and **2** with amines **10**−**15**. The first experiment was to compare pH 11.7 with pH 9.2. As the results show in Table 1, the primary amines **10**, **11**, and **12** reacted more efficiently at pH 11.7 with **1**. The naphthalenemethylamine (**13**) and secondary amines (**14** and **15**) reacted better at pH 9.2 with **2**. The second experiment was to compare pH 9.9 with pH 9.2. The results show that amines **10**−**14** reacted more efficiently at pH 9.9 with **1**, but antibiotic **15** still reacted slightly better at pH 9.2 with **2**. For further comparison, the results of Tables 1 and 2 were normalized. As shown in Table 3, the best derivatization pH for **10** and **11** was 11.7 (buffer A), for **12**, **13**, and **14** it was 9.9 (buffer B), and for **15** it was 9.2 (buffer C).

Stoichiometry. To optimize the stoichiometry of derivatization, a 9 pmol/μL solution of antibiotics **16**, **17**, and **18** in buffer C (pH 9.2) was reacted with varying amounts of **1** from 4 to 44 molar excess. After 10 min, an excess amount of **2** (22x) was added to each reaction to convert any unreacted antibiotic to derivatives of **2**. The relative amount of derivatives with **1** and **2** in each

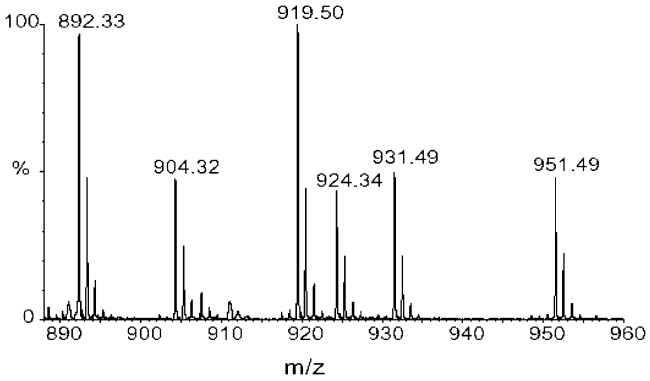

Figure 4. MALDI-TOF spectrum of 750 fmol of **1** and **2** derivatized **16** (C^+ = 892.33 and 919.50), **17** (C^+ = 904.32 and 931.49), and **18** (C^+ = 924.34 and 951.49).

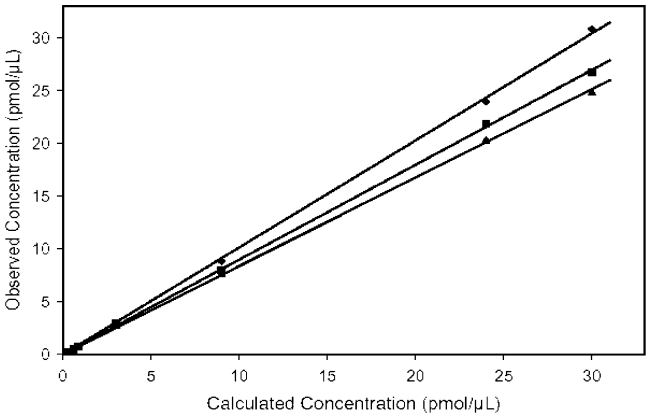

Figure 5. Plots of the observed and calculated concentrations of antibiotics **16** (■, r^2 = 0.9997), **17** (◆, r^2 = 0.9996), and **18** (▲, r^2 = 0.9997) in the test solutions.

reaction was evaluated by MALDI-TOF MS. Equation 4 was used to estimate the percent yield of the coupling reaction when a certain amount of **1** was added. The calculation assumes that the reaction will reach completion when a large molar excess of reagents are used. Figure 3 is a plot of the percent conversion yield of coupling reaction against the molar ratios of **1** to antibiotics. Approximately, a 10*x* molar excess of **1** was sufficient to derivatize **16** and **17** to near completion. However, more than 20*x* molar excess of **1** was required for **18** to achieve the same conversion yield. The difference in reaction yields among the antibiotics may be due to the steric hindrance caused by the methyl group next to the secondary amine of **18**.

Quantitative MALDI-TOF Analysis. The isotopically coded light and heavy reagents (**1** and **2)** were used to explore the potential for quantitative analysis of small analytes by MALDI-TOF MS. The evaluation was carried out in buffer C (pH 9.2) using antibiotics **16**, **17**, and **18** as model compounds. Figure 4 shows the specturm of a mixture of two equal volumes of antibiotic solutions (3 pmol/μL) that were treated with 40*x* molar excess of **1** and **2**. The spectrum shows that the peak intensities of the derivatives of **1** and their corresponding derivatives of **2** are essentially the same. This indicates that antibiotics derivatized by both **1** and **2** have the same cocrystallization behavior with the matrix. To examine the dynamic range of the quantitative analysis by MALDI-TOF MS through derivatization, a series of test solutions containing 0.3, 0.6, 0.9, 3, 9, 24, and 30 pmol/μL (2 orders of magnitude) of antibiotics **16**, **17**, and **18** in buffer C were treated with 40*x* molar excess of **1**. An antibiotic solution containing 3 pmol/μL of **16**, **17**, and **18** in buffer C was reacted with 40*x* molar excess of **2** to form a **2** derivatized antibiotic standard solution (3 pmol/μL). Equal volumes of **2** derivatized antibiotic standard solution and **1** derivatized test solution were mixed, and the resulting solution was analyzed by MALDI-TOF MS. Equation 5 was used to obtain the observed antibiotic concentration (O) in the test solutions.

$$O = \frac{P}{Q} \times (3\ \text{pmol}/\mu\text{L}) \qquad (5)$$

where P is the peak intensity of the derivatives of **1** and Q is the peak intensity of the corresponding derivatives of **2** in each spectrum. Figure 5 shows the plots of the calculated and observed values for the concentration of three antibiotics in the test solutions. The linear correlation coefficients (r^2) for all three analytes are greater than 0.9995, which demonstates the utility of

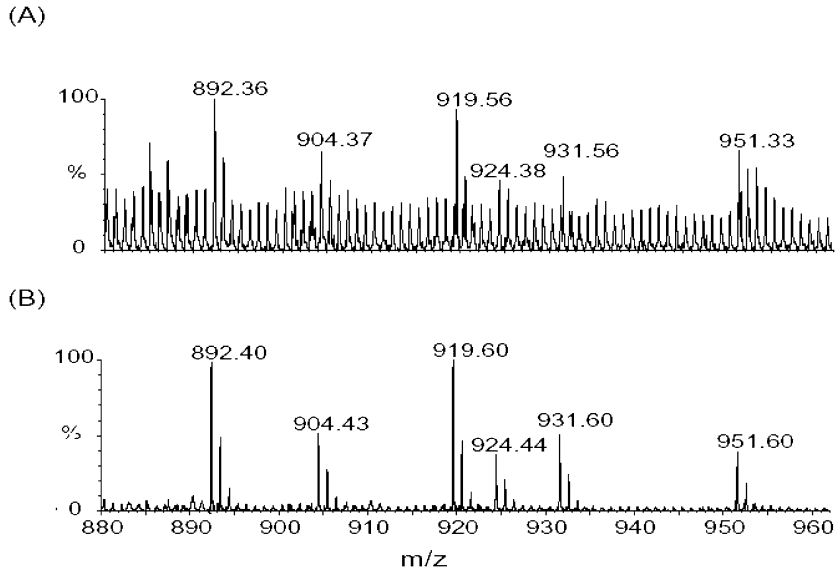

Figure 6. MALDI-TOF spectrum of 3 fmol (A) and 30 fmol (B) of **1** and **2** derivatized **16**, **17**, and **18**.

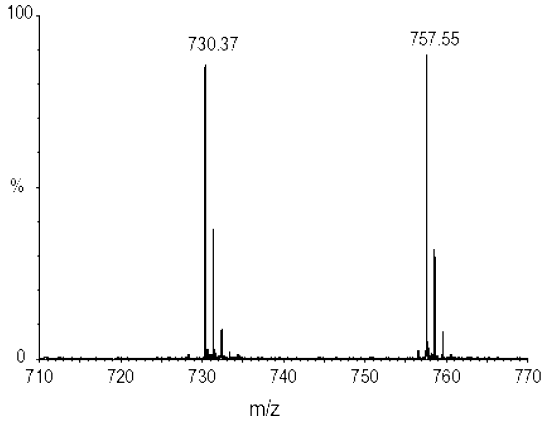

Figure 7. Laser desorption spectrum of 200 fmol **1** and **2** derivatized **13** (C^+ = 730.37 and 757.55) without using a matrix.

quantitative MALDI-TOF MS analysis coupled with derivatization using isotopically coded **1** and **2**.

Although MALDI has a moderate tolerance for contaminants in the sample, it is also well-known that the ionization efficiency can be severely diminished by the presence of salts or buffers in the sample. For any derivatization reaction, there is always a probability of introducing contaminants that may interfere with downstream MALDI analysis. Among all possible contaminantion resources in this study, the primary ones are the derivatizing reagents and the buffers. *N*-Hydroxysuccinimide esters **1** and **2** and their derivatives were observed to be highly compatible with MS analysis, and all the reaction mixtures were analyzed without prior cleanup. Figure 6 shows the MALDI spectra for 3 and 30 fmol of reaction mixture of **1** and **2** derivatized **16**, **17**, and **18** spotted directly on a stainless steel target plate. The limit of detection for this class of compounds is in the low femtomole range. Contrastly, direct MALDI analysis of these antibiotics at picomole loadings without derivatization yielded very weak signals that were buried in the abundant matrix-related peaks (data not shown).

Interestingly, **1**- and **2**-derivatized naphthalenemethylamine (**13**) could be observed without using matrix by direct laser desorption analysis (Figure 7). A high signal-to-noise ratio was achieved at 200-fmol loading. It is presumed that the aromatic nature of the analyte was responsible for this observation.

CONCLUSIONS

Isotopically coded light and heavy *N*-hydroxysuccinimide esters (**1** and **2)** were successfully employed as an aid for analyzing small molecules by MALDI-TOF MS. Esters **1** and **2** rapidly reacted with primary and secondary amines in mild conditions to add a large positively charged tag (573 and 600 Da, respectively) to the analytes. This increased their limit of detection due to improved ionization efficiency and a mass shift away from the matrix clusters. The peak intenisty ratio of derivatives of **1** and **2** obtained from the same MALDI target spot were directly related to the ratio of analyte concentrations in compared samples. It was proven that there is potential for using isotopically coded light and heavy reagents such as **1** and **2** for conducting quantitative MALDI-TOF MS analysis of small molecules and antibiotic mixtures.

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