# Comparison of Sample Preparation Methods and Evaluation of Intra and Inter Sample Reproducibility in Bacteria MALDI-MS Profiling.

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# Comparison of Sample Preparation Methods and Evaluation of Intra- and Intersample Reproducibility in Bacteria MALDI-MS Profiling

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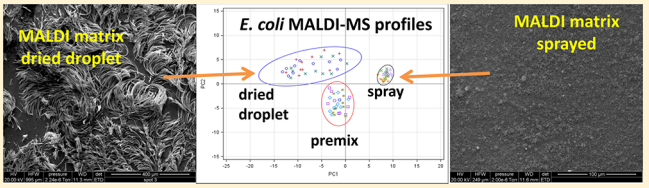
Supporting Information

**ABSTRACT:** A study is presented on the reproducibility of mass spectral profiles of the whole bacterium *E. coli* resulting from laser sampling at different regions within and between matrix-assisted laser desorption ionization (MALDI) samples deposited onto the plate. Samples were prepared with different deposition methods and using different MALDI matrices. The three most common matrices used in MALDI-mass spectrometry (MS) bacteria profiling, α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), and ferulic acid (FA), were compared in this study along with two pipet-based sample deposition methods, dried-droplet and premix. Sample variability was determined by analysis of variances (ANOVA), principal component analysis (PCA), and multivariate ANOVA (MANOVA). For the two pipet-based sample deposition methods tested in this study, the intrasample variability (most commonly referred to as "spot-to-spot" reproducibility) was of the same magnitude as the intersample variability for all MALDI matrices tested. By incorporating a spray nebulizer sample deposition method to produce uniform sample/matrix mixtures onto the MALDI plate, we demonstrate that the crystalline morphology of the MALDI sample greatly influences the intrasample reproducibility (i.e., spot-to-spot) of the resulting whole cell MALDI-MS profiles. Overall, for the pipet-based deposition methods, results showed that the smallest variability in bacteria MALDI mass spectral profiles was obtained from samples deposited using the premix method, regardless of the MALDI matrix used, with the best reproducibility obtained with the CHCA matrix. It is concluded that a sample preparation strategy that reduces or eliminates the MALDI matrix morphology heterogeneity can reduce variability (i.e., spot-to-spot) of the bacteria mass spectral profiles by up to 90%.




Matrix-assisted laser desorption ionization (MALDI)[1,2] has made possible the analysis of large thermally labile biomolecules by mass spectrometry (MS), finding many applications in proteomics,[3−5] nucleic acid detection,[6,7] tissue imaging,[8−10] and intact bacteria analysis.[11−14] In particular, interest in the use of MALDI-MS for the analysis, detection, and identification of intact bacteria in the clinical setting has increased during the past decade. Since its first applications to bacteria analysis, MALDI-MS has been applied to identify and characterize bacteria at the genus, species, and strain levels using both cell extracts[15−17] and whole (intact) bacterial cells.[11,12,18−20] Many attributes make MALDI-MS attractive for the rapid identification of bacteria: (i) the ability to differentiate a wide range of microorganisms (bacteria, viruses, and fungi),[21−25] (ii) the use of minimal sample pretreatment and processing protocols, allowing the direct analysis of intact cells,[16,26−28] (iii) its high sensitivity, with limits of detection (LOD) near the low femtomolar range for peptides and proteins and low picomolar range for DNA,[29,30] (iv) the robustness and reproducibility of the method,[17,25,31−33] (v) the broad mass range covered, (vi) its ability to generate singly charged high mass protein ions which serve as biomarkers for the bacteria identification,[27] and in some instances, (vii) its ability to analyze microbial mixtures.[34−37]

For the analysis of clinical samples, MALDI-MS has been found to provide rapid and accurate identification and characterization of different bacterial strains when compared to currently used phenotype-based bacteria identification systems.[38−43] For example, one prominent study compared conventional biochemical test systems (e.g., enzyme-based assays) with two commercial MALDI-MS systems (MALDI-MS instrumentation and software for bacteria identification) and showed that between 89% and 95% of the bacteria studied were correctly identified by MALDI-MS.[41] In addition, the isolates that showed discordant results with the conventional biochemical tests were resolved with 16S rRNA gene sequencing (used as the reference method), and the results were found to agree with those obtained by the MALDI-MS systems between 75% and 78% of the cases. In addition, this study showed that the MALDI-MS analysis was cost-effective, conclusively demonstrating that MALDI-MS can provide rapid and reliable bacteria identification for routine analysis in clinical laboratories.

Despite the growing application of MALDI-MS in the analysis of clinically isolated bacterial samples, a recent survey of the literature[28,44] shows that MALDI-MS sample preparation protocols are not fully standardized between laboratories,

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especially in the preparation of the matrix/bacteria sample and its method of deposition onto the MALDI plate. It is well-known from early work in the analysis of bacteria with MALDI-MS that strict control of the sample preparation steps was vital for the generation of reproducible mass spectral profiles.[26,45] Even though bottom-up[46] and top-down[47,48] proteomic approaches to bacteria identification can overcome these deficiencies, the sample preparation and instrumentation can be more complex than that required for profile-based measurements and for routine high-throughput clinical work. Because of this susceptibility of the mass spectral profiles to different sample preparation protocols, extensive efforts have been devoted to the characterization of the effect(s) of several experimental variables on the bacteria mass spectral profile obtained with MALDI-MS. Specifically, the experimental variables investigated can be broadly divided between bacteria culture growth conditions[45,49−51] and MALDI-MS experimental conditions. The MALDI-MS experimental conditions investigated include: (i) operator, MALDI sample preparation and instrumentation used,[17] (ii) bacteria cell concentration and reproducibility over time,[18,26,52] (iii) bacteria pretreatment procedures,[52−54] (iv) ionization mode and matrix additives,[52,55] (v) type of MALDI matrix, and (vi) matrix solvents as well as sample deposition methods.[50,52,54,56,57] A comprehensive list of bacterial cell types, pretreatment conditions, and matrix and solvent compositions that have been widely used in MALDI-MS of intact bacteria has been reported.[44] In general, variations in the matrix type, solvent composition (matrix and bacteria suspension), bacteria cell concentration, type of acid, and spotting technique showed significant changes in the mass spectra quality and reproducibility. Further, complicating interpretation of the available data is the known interplay between all these variables. Overall, from these studies, one can generalize that the most widely used matrices are $\alpha$-cyano-4-hydroxy-cinnammic acid (CHCA), sinapinic acid (SA), and ferulic acid (FA), while the most widely used organic solvents are either acetonitrile or methanol in conjunction with either trifluoroacetic acid or formic acid.

The final step in the sample preparation protocol before MALDI-MS detection of bacteria is the sample deposition onto the MALDI probe or plate, and variations in these methods have been reported to also significantly affect reproducibility of mass profiles, the signals detected, and the overall intensities of mass spectra obtained.[58,59] However, it has been also found that a relatively wide range of conditions will yield successful results in MALDI-MS (albeit not reproducible), and thus, this inherent robustness of the MALDI process has led to the development of several sample deposition methods: (1) the dried droplet (DD) or bottom-layer method, which involves depositing an aliquot of bacteria suspension on the target, air-drying it at ambient temperature, and then overlaying an aliquot of matrix solution on the dried sample drop;[33,40,52,57,60] (2) the direct analysis or smear method involves spotting bacteria colonies (i.e., not from a bacteria suspension) directly onto MALDI target, air-drying at room temperature, and then overlaying it with matrix solution;[40,41,52,56] (3) the premix (volume or one-layer) method, in which the bacteria suspension and matrix solution are transferred and mixed in an eppendorf tube by vortexing for a few seconds before depositing the mixture onto the plate and then allowed to air-dry at room temperature.[18,26,45,61,62] This method has been also reported as the dried-droplet method by some investigators;[57] (4) the two-layer (or seed-layer) method, where the sample−matrix mixture is deposited onto a thin layer of predeposited/dried matrix seed;[17,52,57] (5) on-probe premix, a variation of the premix method, where the deposited sample droplet is immediately overlaid with the matrix solution before letting the mixture air-dry at room temperature.[2,39,42,43,51] This method has been also referred to as a two-layer method[63] and as a dried droplet method.[61] Other variations and modifications of these methods have also been employed for MALDI-MS of bacterial cells.

For all of these methods, reports of nonuniform MALDI sample surfaces due to heterogeneity in the crystallization process of the matrix resulted in the need to locate the so-called "sweet" or "hot" spot, a region within the MALDI sample where "high quality" mass spectra can be generated. High quality in this sense can be loosely defined by mass spectra with high S/N, large number of signals, and narrow peaks. The presence of these hot spots may have contributed to the reporting in the published literature of conflicting results as to the optimal type of matrix or sample preparation protocol for MALDI-MS of intact bacteria. In particular, for example, the ferulic acid (FA) matrix has been reported to provide mass spectra with either "enhancement of signal-to-baseline ratio" and high mass signals ($m/z$ > 20 000)[53,64] or poor "shot-to-shot spectral reproducibility" (i.e., within a matrix sample)[50] for the analysis of whole bacteria by MALDI-MS. The most likely cause for this discrepancy (other than instrumental differences cited by Lay et al.[50]) is the prominent heterogeneity of the FA matrix,[58,64] which tends to form large crystals concentrated in a circular pattern on the rim of the dried matrix sample. This heterogeneous morphological characteristic of most MALDI matrices makes the unbiased comparison of optimization methods for sample preparation particularly difficult, as different spots within the sample yield mass spectra of varied "quality". Surprisingly, except for two notable works, one published by Chen et al.[49] comparing mass spectral profiles using a combination of analysis of variances and principal component analysis (ANOVA-PCA)[65] and another by Dong et al.[61] using calculated mean and standard deviations for the base peak intensities, most of the variable optimization and reproducibility studies mentioned earlier relied on subjective nonquantitative means to compare bacteria mass spectral profiles. This strategy is particularly inefficient given that the ability to compare many mass spectral profiles by statistical methods allows for significant and minor variations to be detected, while eliminating possible operator bias in subjectively selecting and/or comparing the overall quality between mass spectra.

Ideally, when using MALDI-MS to identify bacteria, mass spectra are collected from several random regions (i.e., spots) within a single MALDI sample and averaged to yield a mass spectrum used for subsequent database search. To accomplish this without the introduction of operator bias, automated laser sampling options are available in most commercial MALDI-MS systems. However, if the MALDI sample heterogeneity is such that many hot and cold signal regions are prevalent within the sample, the resulting mass spectral profiles may be irreproducible and lead to low confidence matches and/or the inability to differentiate closely related microorganisms (e.g., serologically similar bacteria). In order to characterize the effect of the MALDI matrix/sample heterogeneity on mass spectral profile reproducibility, the present work aims at measuring the reproducibility of the mass spectral profiles resulting from laser sampling at different regions within (i.e., intrasample or spot-to-spot) and between MALDI samples (intersample), for

different matrices and for different sample deposition methods. To show the effect due to the lack of heterogeneity in the matrix morphology to the overall reproducibility of the mass spectral profiles, we incorporate the use of a spray nebulizer sample deposition method to produce uniform mixtures of sample and matrix onto the MALDI plate. The mass spectral profiles resulting from laser-sampling at different regions within and between MALDI samples are compared via pattern recognition (principal component analysis, PCA) for all matrices and sample deposition methods, and conclusions are drawn as to ways to improve the shot-to-shot reproducibility of the MALDI bacteria mass spectral profiles.

## EXPERIMENTAL SECTION

**Chemicals.** The MALDI matrices $\alpha$-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), ferulic acid (FA), trifluoroacetic acid (TFA), and horse heart cytochrome c were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Acetonitrile (ACN) was purchased from Burdick and Jackson (HPLC grade, Muskegon, MI), and water was obtained from a Milli-Q Plus purification system (Millipore Corp., Bedford, MA)

**Bacteria.** The bacteria *Escherichia coli* (K12) ATCC 15597 used in this study was purchased from America Type Culture Collection (ATCC, Manassas, VA) and grown on tryptic soy agar (TSA; BD Science, Sparks, MD) plates at 37 °C for 12−15 h. The cells were harvested using a sterile wire loop and transferred to a 1.5 mL Eppendorf tube containing 1 mL of sterilized deionized water. The cell pellets were vortexed for 30 s and then centrifuged at 10 000 rpm for 1 min to remove any media present. An additional washing step was carried out, and the cells were resuspended in water. The cell concentration was determined by performing a viable cell count which involved serial dilutions of the cells in sterile deionized water and cells grown on TSA plates. The concentration of cells obtained was $4.7 \times 10^8$ cfu/mL (cfu = colony forming units). The bacteria suspension was immediately used for the MALDI-MS experiments.

**Sample Preparation.** In this work, the terminology used is as follows: (1) a *MALDI sample* corresponds to the sample droplet deposited and dried onto the plate and (2) a *MALDI spot* corresponds to a unique set of $x$ and $y$ coordinates where the laser is aimed (within the MALDI sample) and an average mass spectrum is collected (Figure S1 in the Supporting Information section shows a diagram of the terminology used). CHCA, SA, and FA were used as the matrices since they are the most widely used[44] for MALDI-MS of bacteria. A solution of each matrix was made by dissolving 10−12.5 mg/mL in a solution containing acetonitrile, water, and acid. Specifically, CHCA and FA were each dissolved in 50/50 (v/v) of ACN/$H_2O$ containing 2.5% TFA. SA was prepared in a solvent of 50/50 (v/v) ACN/$H_2O$ containing 1% TFA. The concentrations of TFA were chosen so as to match the most common sample preparation protocols currently available in the literature. Cytochrome c was used as a mass internal standard[32] ($[MH]^+$ at $m/z$ 12361) and was adjusted to a concentration of 1−2 $\mu$M in the matrix solutions. Solutions were then applied by each method: dried droplet, premix, and spray deposition, the latter using an oscillating capillary nebulizer (OCN).[66,67] A detailed description of the construction and optimization of the OCN used to spray-deposit the bacteria can be found in the Supporting Information section.

**MALDI-MS Analysis.** MALDI-MS analyses were performed on a MALDI-time-of-flight-MS system (Voyager DE STR, Applied Biosystems, Foster City, CA) operated with the following settings: positive ion, linear mode, delayed extraction time of 400 ns, acceleration voltage of 20 kV, and a grid voltage of 93%. A nitrogen laser operating at 337 nm wavelength with a 3 Hz repetition rate was used, and mass spectra resulting from 50 laser shots were accumulated to obtain an average mass spectrum at each sample spot. For the 3 spot experiments, locations were chosen that visually showed variations in the sample−matrix morphology. For the 10 spot experiment, locations were chosen at random and all resulting average mass spectra were saved (i.e., none were rejected). All mass spectra shown correspond to the average mass spectrum obtained from 50 laser shots. For the spray deposition sample, different spots were equidistant (~200 $\mu$m apart) along the center of the sprayed matrix−sample trace.

**Pattern Recognition.** Principal component analysis (PCA) was performed using a commercially available software package (Pirouette Lite, Ver. 3.11, Infometrix, Woodinville, WA) on the thirty replicate mass spectra collected for each sample deposition method investigated. An in-house MATLAB program was developed that was used to group peaks that occur within a ± 2 Da mass window (linear TOF mass accuracy of 0.05%). The MALDI mass spectra were first processed by setting the peak detection to a base peak intensity of 10% (relative intensity) and then centroid to reduce the number of peaks to be applied in PCA. All PCA data generated were from mass spectra collected on the same day. Data for principal component one (PC1) and principal component two (PC2) were imported into a SAS 9.2 software package (SAS Institute Inc., Cary, NC, USA) and used to generate PCA score plots including the prediction and confidence ellipses around each cluster (mass spectra data for each deposition method) for each matrix at a level of significance of $\alpha$ = 0.05.

## RESULTS AND DISCUSSION

MALDI samples prepared via pipet methods are known to yield different mass spectra (both in intensity and profile) from different spots within a sample. This variation can often mask the effects of other parameters being investigated (e.g., the effect of matrix type or solvent used), and this in turn can lead to operator bias during data acquisition in selecting a "representative" mass spectrum. In this study, in addition of not rejecting any mass spectrum during data acquisition, we incorporate the use of a spray deposition method to achieve a uniform film of the matrix/sample onto the MADLI plate, thus reducing the matrix sample morphology heterogeneity. This reduction in the matrix morphology in turn reduces localized differences in sample concentrations within the MALDI matrix (i.e., signal hot spots), resulting in MALDI-mass spectra with many constant intensities and peak ratios that are independent of the laser sampling spatial coordinates (i.e., eliminates spot-to-spot variations within a MALDI sample). Scanning electron microscopy (SEM) images of the matrix/sample mixture for the dried-droplet deposition method and spray nebulizer deposition are shown in Figures S4 and S5 in the Supporting Information section and illustrate the striking effect of the spray sample deposition method in reducing the matrix morphology heterogeneity.

The effect of spot-to-spot or intrasample variations in the mass spectral profiles due to morphology heterogeneity of the MALDI matrix is illustrated in Figures 1 and 2 for the FA

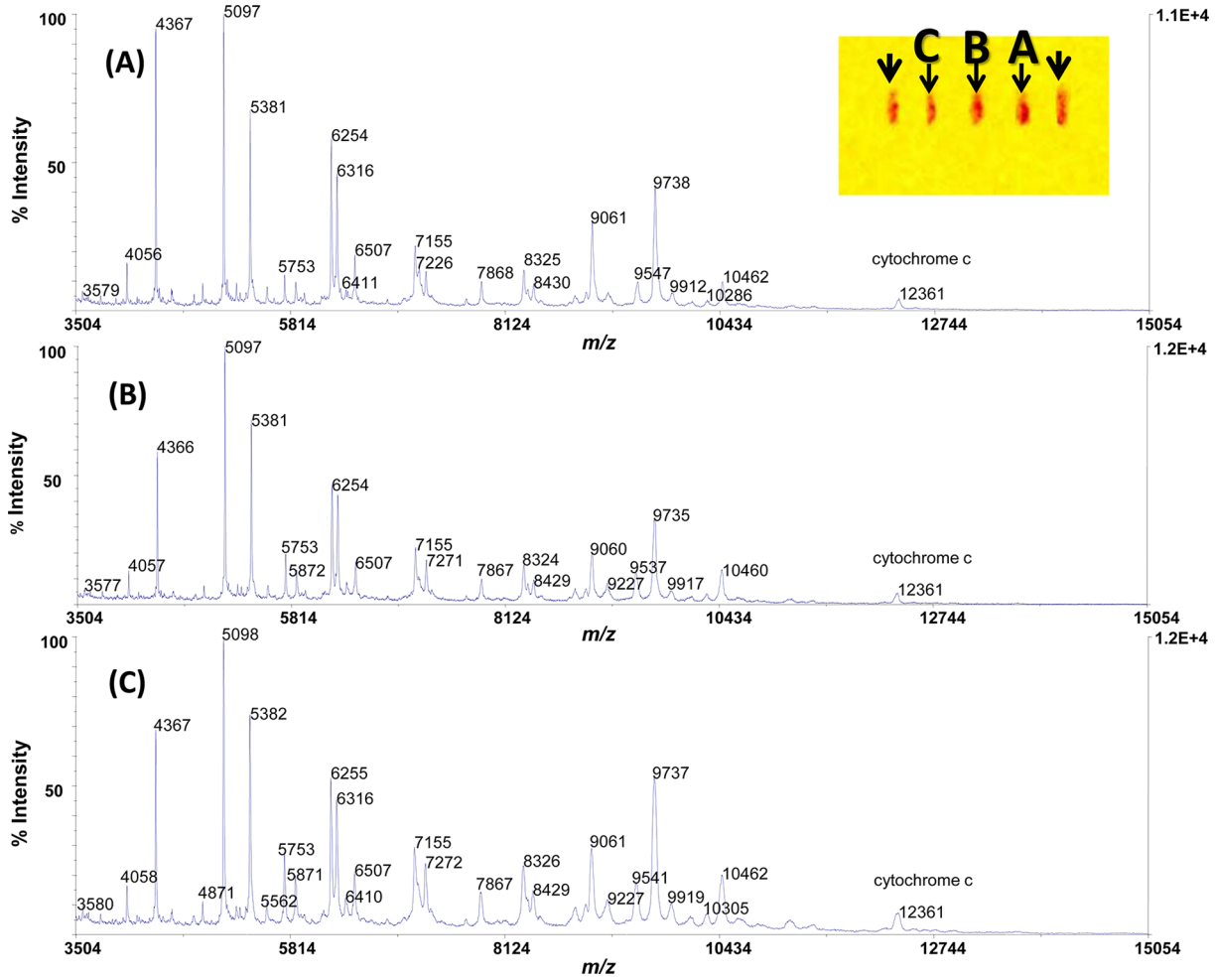


**Figure 1.** Three mass spectra (A, B, and C) obtained at 3 different spots within a single MALDI sample (A, B, and C shown on the inset image) for spray deposition of an *E. coli* (K12) and ferulic acid (FA) matrix mixture.

matrix, where the pipet-based deposition method can be compared to the spray deposition method for the analysis of *E. coli* K12. For the purpose of this illustration, mass spectra were collected at 3 different spots within a MALDI sample prepared by spray deposition (Figure 1) and dried-droplet deposition (Figure 2). The same mass spectra for the premix deposition method are shown in Figure S6 in the Supporting Information section. Each figure also includes a microphotograph (obtained at 60× magnification) of the resulting MALDI sample for the FA matrix and the exact spots (A, B, and C) sampled to produce each of the three replicate mass spectral profiles shown. The mass spectra obtained from the sprayed sample using the FA matrix (Figure 1) produced mass spectra with reproducible overall signal intensities and ratios at the spots sampled (A, B, and C), thus demonstrating a high degree of spot-to-spot reproducibility. On the other hand, the mass spectra obtained from the dried-droplet method (Figure 2) showed a high spot-to-spot signal intensity variation, including that of the internal standard signal at $m/z$ 12 361. For this MALDI sample, the highest absolute signal intensity was obtained at a spot near the edge (C) of the MALDI sample. In addition, several mass spectral peaks (between $m/z$ 7000 to 10 000) either are not present or have different peak ratios between the mass spectra. The same spot-to-spot measurements for each deposition method were also obtained for the CHCA and SA matrices (with 3 replicate spots probed from MALDI samples prepared by each method) and the resulting mass spectral profiles are shown in Figures S7−S9 (for CHCA) and Figures S10−S12 (for SA) in the Supporting Information section. Collectively, these data qualitatively demonstrate that matrix morphology heterogeneity is a major contributor to the spot-to-spot variability in signals obtained within a MALDI sample and that the spray deposition method is an effective way to decrease this variability. Although not studied in much detail, high mass measurements (9000−30 000 u) were also performed for the FA and SA matrices since they are better suited for the analysis of large molecular weight (MW) proteins. Preliminary results show no clear advantage in terms of more high MW protein signals detected with the spray method (data not shown), although reproducibility is clearly improved (vide infra).

**Effect of Sample−Matrix Morphology on Intact Bacteria MALDI Mass Spectral Signal Intensity and Reproducibility.** To make a quantitative assessment of the sample-to-sample (intersample) and spot-to-spot (intrasample) signal variability in MALDI mass spectral profiling of bacteria, a larger mass spectral data set was studied. Specifically, mass spectral profiles were obtained for *E. coli* K12 bacteria using the dry-droplet and premixed deposition methods (in addition to the spray deposition method) for the MALDI matrices CHCA, SA, and FA. In all, a total of 30 mass spectra were collected for each matrix and deposition method (i.e., 3 different MALDI


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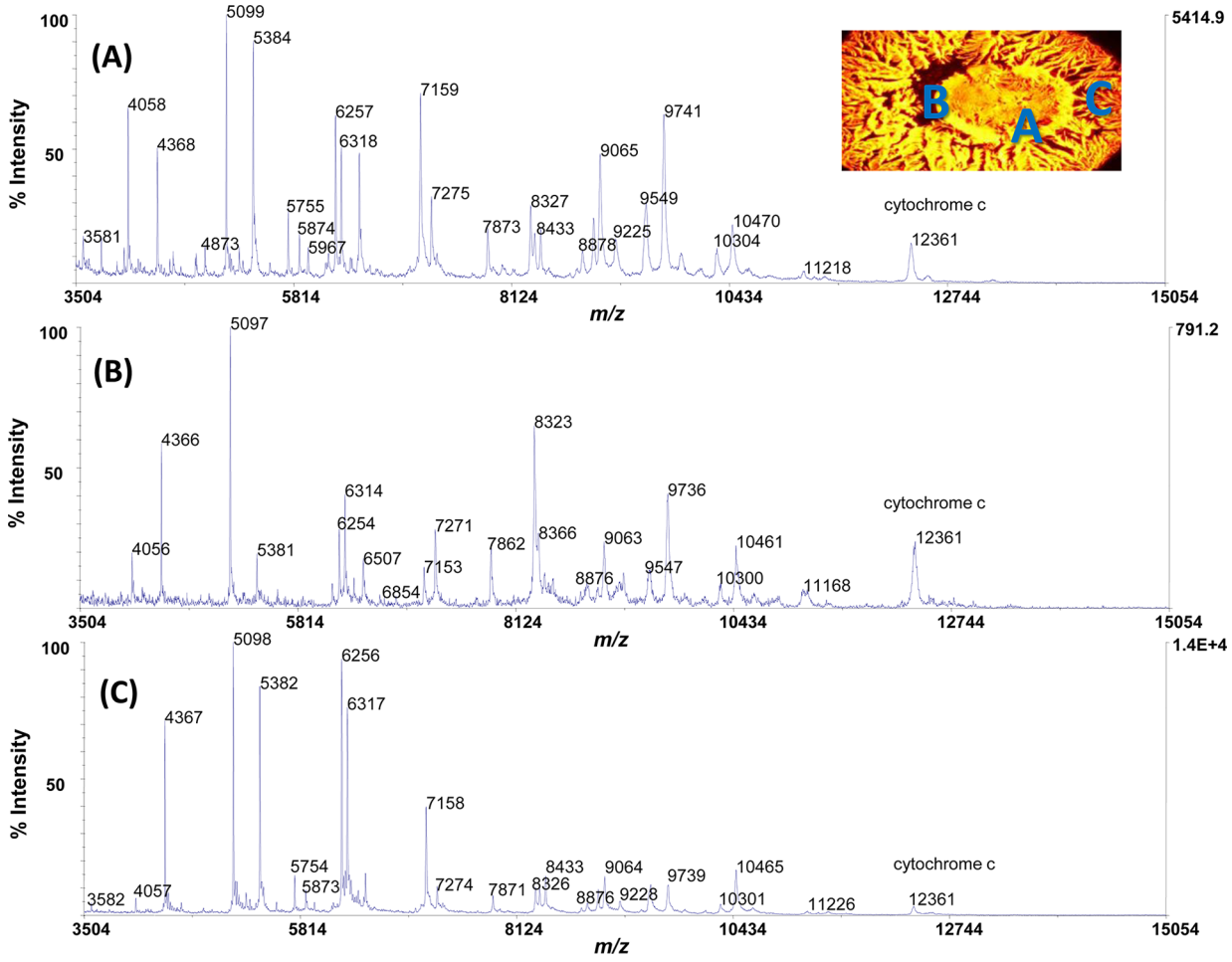


**Figure 2.** Three mass spectra (A, B, and C) obtained at 3 different spots within a single MALDI sample (A, B, and C shown on the inset image) for a dried-droplet deposition method: *E. coli* (K12) suspension was first deposited and then overlaid with FA matrix.

samples each probed at 10 different spots). For this data set, the effect of sample deposition and matrix type on factors like absolute signal intensity (peak areas), signal-to-noise ratio (S/N), and peak resolution was investigated for the signals at $m/z$ 4366 ± 2 and $m/z$ 9062 ± 2 present in the *E. coli* mass spectrum. Histograms in Figure 3 summarize results for these measurements. Inspecting Figure 3a, it can be seen that the percent relative standard deviations (% rsd) range between 20% and 69% for the pipet-based methods and for all matrices, while the spray method, which is mostly devoid of heterogeneous matrix morphology, yielded variations between 6% rsd and 12% rsd for this signal. The lowest % rsd for the signal at $m/z$ 4366 for the pipet-based methods is obtained for the CHCA matrix using the premixed deposition method. The dried droplet deposition method consistently yielded the highest % rsd values in the signal intensities with values between 47% rsd and 69% rsd for all matrices tested. Inspection of the variations in S/N rather than absolute signal intensity (Figure 3b) reveals a similar trend, with the CHCA matrix deposited using the premixed method showing the smallest % rsd. However, the CHCA matrix using the premixed sample deposition also showed the smallest absolute S/N ratio for the signal at $m/z$ 4366. Both the FA and SA matrices yielded the highest absolute S/N values when used with both pipet-based methods; albeit, they also yielded the highest % rsd values (30−55% rsd). From this last result, it is feasible to anticipate a situation where any of these matrices (FA or SA) could in fact appear to outperform CHCA solely based on terms of improved mass spectrum S/N; that is, producing a mass spectrum of higher "quality" without taking into account its spot-to-spot reproducibility. (For example, see Figure S12 in the Supporting Information section. These mass spectra obtained with the SA matrix can be considered of very high quality; however, the SA matrix showed one of the highest standard deviations for peak intensities (see Figure 3a) and largest profile deviations (vide infra Figure 4c and Table S5 in the Supporting Information).) These observations may explain in part the wide range of matrix preferences for bacteria mass spectral profiling in the published literature, ranging from SA[32,49,60,68] to CHCA[50,52] to FA.[33,53] Our observations are also in agreement with results presented by Musser et al. in a thorough optimization study[50] where they noted poor shot-to-shot (i.e., intrasample) reproducibility for the FA matrix when compared to CHCA and using the premix deposition method. Finally, from inspecting Figure 3, it can be noted that the spray deposition method yielded highly reproducible peak areas and S/Ns, with % rsd ranging from 6% rsd to 17% rsd for all matrices tested, with the lowest % rsd obtained when using the CHCA matrix. This % rsd trend is also true for signals above $m/z$ 9000 (Figure 3c) and with signal intensities being highest for the SA matrix using the premix deposition method, although it also showed the largest standard deviation. On the other hand, the mass spectral resolution was found to be mostly unaffected (within experimental error) by


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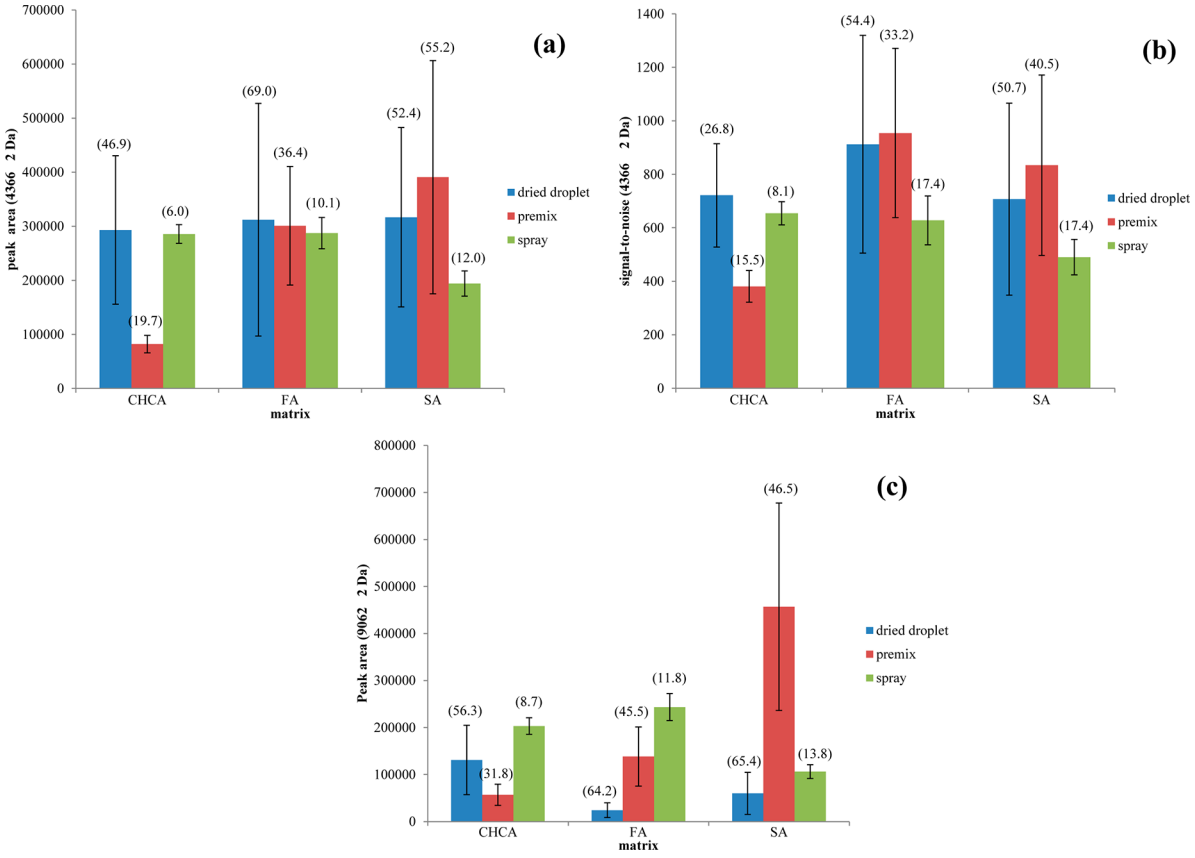


**Figure 3.** (a) Peak areas and (b) signal-to-noise ratio (S/N) for the peak at $m/z$ 4366 ± 2 in the *E. coli* (K12) mass spectrum with the matrices CHCA, FA, and SA and deposited using the dried-droplet, premix, and spray methods. (c) Peak areas for the signal at $m/z$ 9062 ± 2 (For all plots: error bars represent $\pm 1\sigma$ for $n$ = 30; % rsd in parentheses).

the type of matrix and pipet-based deposition method (see Figure S13 in the Supporting Information section).

Using analysis of variances (ANOVA, Table S1 in the Supporting Information section) on the data in Figure 3, it can be concluded that: (i) there are significant variations in peak intensities (i.e., shot-to-shot) within samples that were deposited using the dried droplet method for all matrices tested. This variation within a sample was found to be of the same magnitude as the variations between samples, (ii) there is a significant variation in signal intensities among samples deposited with the premix method when using the FA and SA matrices but not with the CHCA matrix, (iii) there is no significant difference between samples that were deposited with the spray method, regardless of the matrix tested (FA, SA, and CHCA), and finally (iv) for the spray deposition method, the variations within a sample are smaller than between samples.

Overall, the results presented show substantial evidence that matrix morphology heterogeneity within a MALDI sample has a direct effect on the reproducibility of the signal strength and S/N in bacteria MALDI-mass spectral profiles. In fact, by comparing these results with those obtained using the spray deposition method, the contribution of the matrix morphology heterogeneity to the total variance ($s^2$) of the signal intensity can be approximated, and it was found to contribute more than 90% of the total variance of the measurement (see the Supporting Information section for details of this calculation and Table S2 for summary of the calculations). This study, however, does not take into account variances in the signal intensity due to sample growth conditions, which were shown by Harrington et al. to be the major contributor to the variance in MALDI-MS bacteria profiling.[49] Since the utility of the whole bacteria MALDI-MS analysis relies on the comparison of the mass spectral profiles to identify and differentiate bacteria, the effect of this heterogeneous matrix morphology on the overall intact bacteria MALDI mass spectral profile was investigated and quantitatively characterized in a similar fashion.

**Effect of MALDI Sample Matrix Morphology on the Reproducibility of Intact Bacteria MALDI-Mass Spectral Profiles.** To evaluate the reproducibility of mass spectral profiles as a result of matrix morphology heterogeneity, we compared a total of 30 replicate mass spectral profiles obtained for each deposition method and matrix; that is, 10 spots within 3 different MALDI samples were analyzed. Figure 4 shows the principal component analysis (PCA) score plots for all mass spectral profiles obtained with each deposition method and for each matrix (a total of 90 mass spectral profiles per PCA score plot). Also shown in the score plots are the 95% *prediction* ellipses around the different clusters of mass spectra (PCA score plots with the corresponding 95% *confidence* ellipses for the data are shown in the Supporting Information, Figure S14). These 95% prediction ellipses are regions for predicting a new observation in the population and approximate a region containing 95% of the population. Also, indicated with similar symbols in Figure 4 are the different mass spectral profiles that were obtained at different spots within a MALDI sample (i.e., shot-to-shot); that is, similar data symbols represent mass spectra obtained within the same MALDI sample. The clusters


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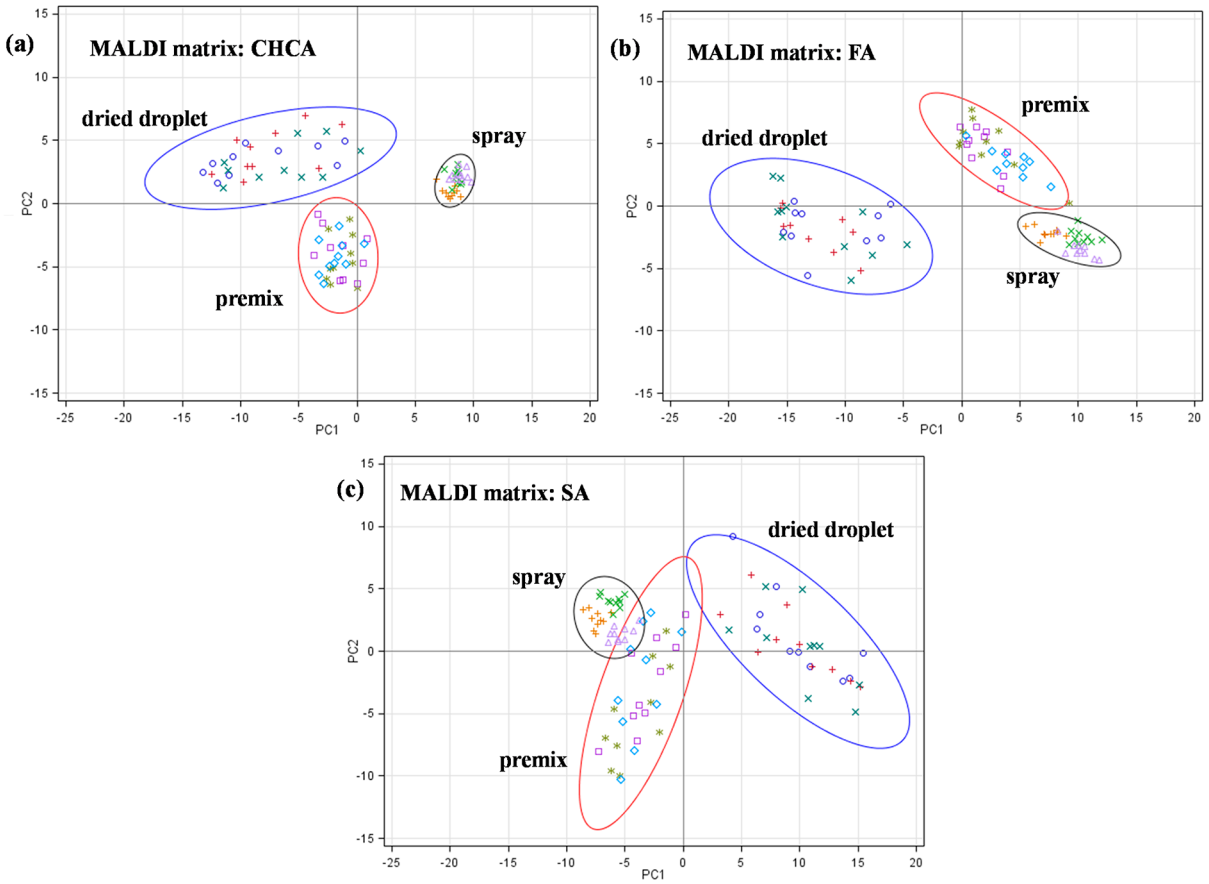


**Figure 4.** PCA score plots with 95% prediction ellipses for the MALDI matrices (a) CHCA, (b) FA, and (c) SA. MALDI-mass spectral profiles compared in these PCA score plots were the results of 30 replicate measurements for spray, premix, and dried droplet sample deposition methods using *E. coli* (K-12) as the bacteria. Similar data symbols represent replicate mass spectra obtained at different spots within the same MALDI sample.

in Figure 4a–c occupy different regions within the score plots, an indication of differences in the peak ratios and signal strengths between the mass spectral profiles obtained by each deposition method for each matrix.

To quantify the spread of the clusters in the score plots in Figure 4, the areas for the 95% prediction ellipses as well as the square root of the determinant of the covariance matrices, $|\Sigma_c|^{1/2}$, were calculated and listed in Tables S5 and S6 in the Supporting Information. The square root of the determinant of the covariance matrix ($|\Sigma_c|^{1/2}$) is a measure of the area of the distribution and is proportional to the area of the ellipse encircling all the mass spectral profiles within one standard deviation of the mean profile.[69] Results in both Figure 4 and Table S5 in the Supporting Information indicate that reproducibility in the mass spectral profiles is worst with the dried droplet deposition method and improves with the premix deposition method, regardless of the matrix used. The improvement in reproducibility due to sample deposition method (dried droplet vs premix) is most notable for the CHCA and FA matrices with ~85% reduction in the ellipse areas and is least notable for the SA matrix with only a 29% reduction in the ellipse area. It is important to note that the SA matrix yielded the largest variations in mass spectral profiles regardless of the pipet-based methods used, when compared to the other MALDI matrices tested. Furthermore, $|\Sigma_c|^{1/2}$ values for the pipet-based methods (dried droplet and premix) for all matrices tested are about 2.5 to 11 times larger than those obtained for the spray deposition method, clearly indicating an increase of the mass spectral profile reproducibility through the elimination (or considerable reduction) of the matrix morphology heterogeneity. Results in Figure 4 and Table S5 in the Supporting Information also point to the CHCA matrix as yielding mass spectral profiles with the smallest variation for the pipet-based methods, with the premix method showing the smallest overall variation for the pipet-based methods. The SA matrix, when used with the premix deposition method, on the other hand, produced mass spectral profiles with considerably higher variations than both the CHCA and FA matrices. Overall, the SA matrix showed the highest variability in the mass spectral profiles regardless of sample deposition method.

For the spray deposition method, where matrix morphology heterogeneity is reduced considerably, the spot-to-spot variability is reduced relative to the pipet-based methods. Also worth noting is that areas of the ellipses for the spray deposition method change with matrix type, albeit by a factor of 2 or less, indicating that the reproducibility of the spray method is not completely independent of matrix type. Specifically, spray-deposited samples with the CHCA matrix yielded mass spectral profiles with the least variability followed by the FA and SA matrices. We speculate that this trend is still dependent on matrix crystal morphology as supported by the SEM images shown in Figure S5 in the Supporting Information (obtained at 10 000× magnification), where the surface morphology of the spray-deposited matrices is most homogeneous for CHCA and least for the SA and FA matrices.


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Finally, a Hotelling's $T^2$ test and multivariate ANOVA (MANOVA) were performed on the PCA data to test the equality of the mean vectors between two groups (methods) for each matrix. Results show (Table S4 in the Supporting Information section) that significant differences exist between mass spectra profiles obtained for all three methods and matrices. This is yet another indication that sample preparation conditions must be strictly controlled in order to yield comparable, highly reproducible, and database searchable mass spectral profiles.

**Inter- and Intrasample Variations in Bacteria MALDI Mass Spectral Profiles.** The inter- and intra-MALDI sample variances for each matrix and each deposition method were determined by comparing the resulting square root of the determinant of the covariance matrices, $|\Sigma_c|^{1/2}$, for the same data set in Figure 4. Table S7 in the Supporting Information shows the $|\Sigma_c|^{1/2}$ values obtained for each of the three sample deposition methods (D, P, and S) with three replicate MALDI samples (1, 2, and 3) for the CHCA matrix. The intrasample $|\Sigma_c|^{1/2}$ values represent a measure of the variations within a MALDI sample (i.e., spot-to-spot variations) for each of the 3 samples prepared by each method. It was observed that, for a particular deposition method, these variations within a MALDI sample were relatively constant for different MALDI samples. Moreover, for the pipet-based methods (D and P), this variation is smallest for the premix (P) deposition method. Also, for these two pipet-based methods, the values for the intersample $|\Sigma_c|^{1/2}$ and intrasample $|\Sigma_c|^{1/2}$ are similar in magnitude, indicating that variations from bacteria mass spectral profiles obtained from within a single MALDI sample (i.e., different spots within a MALDI sample) are similar in magnitude to the variations of profiles obtained between different MALDI samples (dried droplet being 5.39 versus 5.00 and premix being 2.17 versus 2.07). If, however, the effect of matrix morphology is removed or considerably reduced, as in the spray deposition method, the intrasample variations (i.e., spot-to-spot variations) are reduced by nearly a factor of 2 when compared with the intersample variations (average intrasample $|\Sigma_c|^{1/2}$ = 0.26 vs intersamples $|\Sigma_c|^{1/2}$ = 0.48). The same trend in inter- and intrasample variances are observed for FA and SA matrices, and a summary of these results are shown in Table S6 in the Supporting Information section.

## CONCLUSIONS

Our findings demonstrated that matrix surface morphology heterogeneity is an important factor contributing to mass spectra profile reproducibility in bacteria MALDI-MS analysis. By comparing mass spectral profiles from samples prepared by two pipet-based deposition methods (premixed and dried droplet) and the spray deposition method, we have shown that the most homogeneous MALDI sample surface yields more reproducible bacterial MALDI mass spectral profiles. For the pipet based methods, the intersample variance was found to be similar to the intrasample (spot-to-spot) variance, while for the spray method, which reduced the spot-to-spot variability, the intrasample variance was found to be half of the intersample variance. For the pipet-based deposition methods, the premixed method with either CHCA or FA was shown to produce the most reproducible mass spectra profiles (see Table S5 in the Supporting Information). However, the lowest overall signal intensities were obtained for the premix method using CHCA, so this should also be considered in applications where high sensitivity and low detection limits are required. It is important to stress that this study focused solely on the effect of several MALDI sample preparation protocols on the *reproducibility* of the mass spectral profiles and not on the overall "quality" of the profiles. This observed variation in mass spectral reproducibility, and often quality, with highly heterogeneous matrix samples can lead to the occasional production of high S/N (i.e., high quality) mass spectral profiles that often mask the poor reproducibility (intrasample) in a series of mass spectral profiles. This fact can lead to operator bias and skewed results, especially when the resulting mass spectrum is to be compared against a mass spectral database. Overall, the smallest magnitude of spot-to-spot variability in signal intensity and profile was obtained for the premix method and CHCA matrix. For the two pipet-based methods as a whole, the CHCA matrix showed the least variability in mass spectra profiles as compared to FA and SA. In addition, for all matrices, the spray method practically eliminated matrix hot spots and spot-to-spot variability such that mass spectra showed improved reproducibility relative to the pipet-based methods tested (~85% cluster size reduction as measured by PCA). However, in order to take advantage of the high reproducibility afforded by the spray deposition method, it will be essential to incorporate an inactivation step before the spray deposition in order to kill any vegetative or sporulated cells to make this method useful and safe for the analysis of pathogenic bacteria and/or clinical samples. Lastly, the results presented here also raise the question of the associated effect that reducing the matrix morphology heterogeneity (to increase the reproducibility) has on the number of features in the mass spectral profiles (or mass spectra quality). That is, is there a concomitant enhancement (or reduction) of unique mass spectral features that enhance the differentiation between bacteria? Although outside the scope of this study, this is an important question that must be addressed in a systematic way by comparing many mass spectral profiles derived from closely related bacteria.

## ASSOCIATED CONTENT

### Supporting Information

Additional material includes detailed experimental procedures and OCN construction and optimization as well as additional mass spectral profiles and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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