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Sample Depletion of the Matrix-Assisted Laser Desorption Process Monitored Using Radionuclide Detection

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To investigate analyte consumption during the laser desorption process, matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) is combined with radionuclide detection. Radionuclide detection provides highly sensitive and quantitative information on the amount of radiolabeled analytes in a MALDI MS sample spot. ^{14}C-Labeled cytochrome *c* is deposited with 2,5-dihydroxybenzoic acid in 10-nL volume spots. By comparing radioactivity levels of the labeled cytochrome *c* both before and after spectral acquisition, the reduction in labeled analyte molecules on the target allows monitoring of the moles of desorbed sample. Through a depletion study on this sample, the amount of analyte consumed for MALDI time-of-flight spectral acquisition and the average number of molecules desorbed per laser ablation are determined. When [^{14}C]-cytochrome *c* is no longer detected by MALDI MS, ∼70% of the original analyte remains in the sample spots. Redissolving the spots produced further desorption, indicating that the analyte before dissolution was in a physical environment that did not facilitate the desorption process. As a technique with a response that does not depend on the environment of the analyte, radionuclide detection allows characterization of mass-limited sampling methods to better understand the MALDI process.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) was introduced in the late 1980s,[1,2] and it has greatly expanded the utility of MS for the analysis of biological samples. MALDI is a widely used soft ionization method that allows desorption and ionization of high-mass analytes with minimal fragmentation.[3] The benefits of this analytical technique are demonstrated in the wide variety of samples that have been assayed by this method: enzymatic digests,[4] crude food extracts,[5] human breast cancer cells,[6] whole-cell lysates of bacteria,[7−12] peptides and proteins,[13−16] single neurons, and neuronal subsections.[17−22]

MALDI can be considered a relatively nondestructive ionization technique. A sample spot on the target can be assayed multiple times because only a small fraction is vaporized for each laser pulse as compared to electrospray ionization, where the sample is lost once introduced into the instrument. However, no further spectra are detected after repeated ablation by the laser in MALDI. Given the trend to use thinner films and smaller spots,[23−27] a quantitative understanding of sample depletion is important. However, the difficulty in using MALDI MS for absolute quanti-

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tation makes such studies challenging.[28−31] To gain a better understanding of the MALDI process and issues related to sample depletion, a unique scheme of combining quantitative and sensitive radionuclide detection and MALDI MS has been developed.

A radiolabeled analyte is prepared on a target and the radioactivity recorded by a phosphor-imaging detector. The target is then mass analyzed, and the radioactivity in the spot is recorded again, providing a quantitative account of the sample consumption for that spectrum acquisition. This provides information on how much sample is consumed when a mass spectrum is acquired and also gives, on average, how much sample is used per laser ablation. Since the detection limits of radionuclide detection are orders of magnitude lower than MALDI MS,[32,33] the amount of analyte still present on the sample target can be determined after it is no longer observed by MS.

We have previously shown the impressive sensitivity in radionuclide detection-coupled analytical techniques[32,33] and the ability of CE/MALDI MS combined with radionuclide detection for assaying single neurons.[34] We demonstrate here the combination of MALDI MS with radionuclide detection to determine the depletion of ^{14}C-labeled cytochrome *c* deposited with 2,5-dihydroxybenzoic acid (DHB) in nanoliter-volume spots. In one sample spot, we measure that 57 fmol of cytochrome *c* is depleted during the acquisition of the first mass spectrum. On average, 110 amol of cytochrome *c* is ablated per laser shot. Interestingly, when the sample spot is depleted so that the cytochrome *c* signal is no longer observed by MALDI MS, 71% of the original sample is still left in the deposited spot. Using this approach, we hope to obtain a better understanding of the desorption/ionization process. Although much research has been done to characterize this MS ionization technique,[35−37] a complete understanding has not yet been achieved. While most studies use MS or microscopic imaging techniques to evaluate the desorption process,[35,38,39] radionuclide detection offers the advantage of robust and quantitative information whose response is independent of the environment of the analyte.

EXPERIMENTAL SECTION

Reagents. The radioisotope-labeled cytochrome *c* was purchased from American Radiolabeled Chemicals Inc. (cytochrome *c* [methyl-^{14}C] methylated; Catalog No. ARC 427, St. Louis, MO). The MALDI matrix used for these experiments was a 2,5-dihydroxybenzoic acid (ICN Biomedicals, Aurora, OH) solution in water from a Milli-Q purification system (Millipore, Boston, MA) with 0.1 vol % trifluoroacetic acid (TFA; Aldrich Chemical, Milwaukee, WI). The [^{14}C]-cytochrome *c*/matrix solution was made by combining 2.0 μL of the [^{14}C]-cytochrome *c* solution with 6.7 μL of a 19 mg/mL DHB matrix solution in a small plastic screw cap vial. The handling and disposal of ^{14}C-labeled cytochrome *c* requires safe-handling procedures as outlined in each institution's division of environmental health and safety guidelines.

Nanoliter-Volume Spot Deposition. To precisely deposit 10-nL-volume sample spots, a computer-controlled fraction collector was used.[40] This home-built system used three Newport motion actuators (Newport Corp., Irvine, CA) controlled by a computer running a program in LabView software (National Instruments, Austin, TX) to provide micrometer precision in the *x*, *y*, and *z* planes. This fraction collector deposited the spots directly onto a standard gold-plated MALDI target (Applied Biosystems, Framingham, MA). The samples were spotted onto the target by use of a 50-μm-i.d./150-μm-o.d. capillary (Polymicro Technologies, Phoenix, AZ). The solution was delivered to the outlet of this capillary for deposition by a syringe pump (Harvard Apparatus, South Natick, MA) running at a flow rate of 40 nL/min. To perform the deposition, a 1.0-μL Hamilton syringe (Reno, NV) was loaded with the sample solution. The end of the needle was then connected to the inlet of the capillary by use of a ~0.5-cm length of Micro-Line tubing (0.010-in. i.d./0.030-in. o.d.; Thermoplastic Processes Inc., Sterling, NJ). By butting the ends of the needle and the capillary together, a low dead-volume union is made. The deposited solution was then collected in 15-s-interval spots by use of the fraction collector. In this manner, ~400 fmol of [^{14}C]-cytochrome *c* was deposited in each spot. The 10-nL-volume sample/matrix spots were then allowed to dry under ambient conditions and produced a ~400-μm-diameter spot of fully mixed sample and DHB.

MALDI MS Analysis. The deposited sample spots were mass profiled using a Voyager DE STR time-of-flight mass spectrometer with delayed ion extraction (Applied Biosystems). Mass spectra were obtained using a nitrogen laser (337 nm) as the desorption/ionization source. The instrument was used in linear mode with an acceleration voltage of 20 kV and a delayed extraction of 350 ns. The grid voltage was set at 95% and the guide wire voltage at 0.1% of the acceleration voltage. The mass spectra were acquired using 500 laser pulses. In all cases, the ~200-μm-diameter laser spot was manually scanned over the sample field so all areas received significant laser fluence for each 500-pulse sample acquisition. A mass calibration was done internally on the target using the known mass of the radioisotope-labeled cytochrome *c*.

Radionuclide Detection. The radioactivity in the deposited sample spots was analyzed using a Fujix BAS 1000 phosphor imager (Fuji Photo Film Co., Tokyo, Japan). The MALDI target containing the sample spots was placed directly on a Fuji BAS-IIIs imaging plate and exposed for 24 h. The imaging plate was then scanned by the instrument and the image acquired. The total recorded radioactivity in each spot was summed and background subtracted using the Fujix BAS 1000 software.

RESULTS AND DISCUSSION

For this study, ^{14}C-labeled cytochrome *c* was chosen as the analyte as cytochrome *c* is a popular calibration protein whose molecular weight falls in the middle-mass range of MALDI MS. This sample was deposited mixed with DHB matrix in 10-nL-

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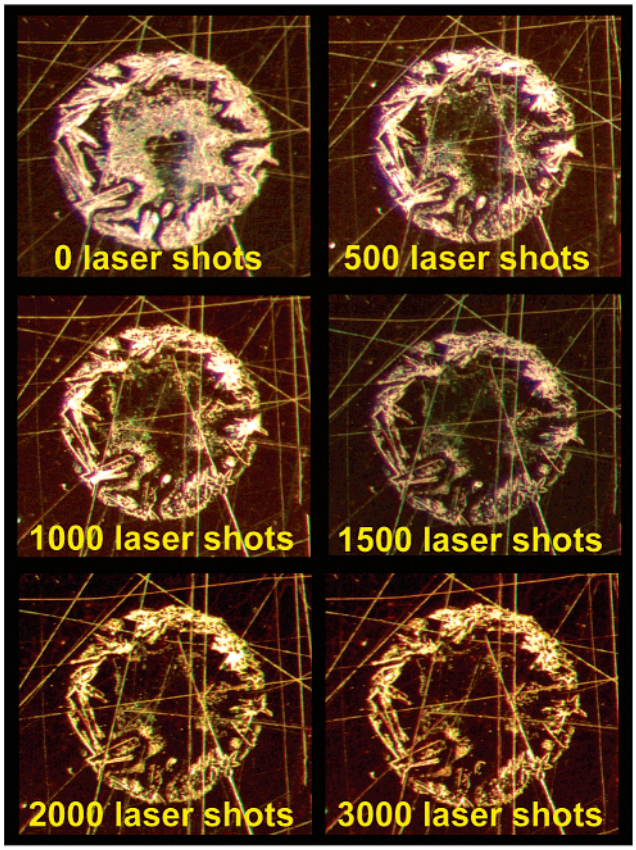

Figure 1. Micrographs of a dried 10-nL sample spot containing DHB and [^{14}C]-cytochrome *c* before and after a series of mass spectra were acquired by MALDI MS. The depletion of the matrix can be seen visually.

volume fractions providing ~400-μm-diameter samples. The reasons for using small sample spots are threefold. First, there has been much attention directed at mass-limited analyses in MALDI MS, and this has resulted in using smaller sample spots and thinner matrix/analyte films. The sample depletion data can be used to optimize these mass-limited analyses. Second, the smaller spot results in less radionuclide usage and radiation exposure. Third, detecting the change in the level of radiation from a single spectral acquisition is more precise. To accurately deposit a series of 10-nL-volume spots onto a gold-plated MS target, an automated fraction collector was used.[40] This provided micrometer positioning of the end of a capillary supplying the matrix/analyte solution at nanoliter per minute flow rates from a syringe pump.

The changes in the appearance of one sample spot from a series of spectral acquisitions are shown in Figure 1. Here, the upper-left image, obtained from a CCD camera mounted to a stereomicroscope, is of the spot before a mass spectrum was acquired. Good-quality needlelike crystals of the DHB can be seen. The image directly to the right of this is of the same spot after a mass spectrum was obtained that was an average of 500 acquisitions (500 laser shots). A decrease in matrix can be observed in the loss of crystals around the perimeter and inside the spot. This decrease continues through four more spectra acquisitions spanning 3000 total laser shots. By examining the first image versus the last image, a considerable loss of matrix is obvious. The film of matrix in the center of the spot is gone, and the crystals making up the perimeter have become thinner and hollow. This shows that when small sample spots are deposited, the matrix is consumed at a rate where the depletion can be seen visually after several thousand laser shots.

The depletion of the sample after mass spectra acquisition is also observed by MALDI MS. Figure 2 shows MS data from two different spots with a column dedicated to the spectra from each spot for various numbers of acquisitions. The top shows the first mass spectrum obtained, which is an average from the first 500 laser shots with the [^{14}C]-cytochrome *c* peak labeled. The second spectrum consists of the second 500 laser shots (1000 laser shots total on the sample); the intensity has dropped considerably. This pattern continues with each successive mass spectrum until the analyte is no longer detected by MALDI MS. This shows that sample depletion occurred rather rapidly. Questions not addressed by physical observation of the spot or by MALDI MS include the following: was all the analyte consumed? If not, how much is still present? Is sample still consumed even though no peak is detected by MS?

We can answer these questions by looking at the data from the radioactivity of the spotted fractions. Figure 3 shows the radioactivity in eight spots at different times throughout the course of the depletion study. Spots 3−8 were subjected to laser irradiation (MALDI) followed by radionuclide detection while spots 1 and 2 were measured only by radionuclide detection but not MALDI. The points along the lines indicate when the mass spectra were acquired. For instance, the points at 0 laser shots are before a mass spectrum was obtained. The next set of data points occurs after 500 laser shots as the first spectral acquisition used 500 laser shots. As the radioactivity corresponds directly to the amount of labeled analyte, the counts of radiation can be converted to moles by using a calibration. The activity of the [^{14}C]-cytochrome *c* sample was provided by the supplier, and a ^{14}C test strip of known activity was used to correlate the arbitrary counts from the phosphor imager to moles (data not shown). Along the *y*-axis is the amount, in femtomoles, of the [^{14}C]-cytochrome *c* in the sample spot. Spots 1 and 2 are from sample spots in which no mass spectra were acquired; the constant values indicate that the depletion was caused by the laser ablation during spectrum acquisition and not the radioactivity measurement process. The dashed line represents the point in the study when [^{14}C]-cytochrome *c* was no longer detected by MALDI MS.

Several types of information can be extracted from this plot. Obviously, the reproducibility of the nanoliter deposition method is obtained: 9.92 ± 0.53 nL. Second, the variance in the radionuclide detection is 0.9% as calculated by the data shown as spots 1 and 2 in Figure 3. Third, the amount of analyte consumption per spectrum acquisition can be obtained. For instance, by looking at the data from the sample spot represented by spot 3, it is calculated that 57 fmol was used to acquire the first spectrum. Also, on average 110 amol of analyte was ablated per laser shot. Fourth, the rate of sample desorption/ionization decreased dramatically as more spectra were acquired. Most surprisingly, when the [^{14}C]-cytochrome *c* peak was no longer detected by MALDI MS there was still 71% of the analyte left in the sample spot that could be observed regardless of laser position or intensity. Essentially, 71% of the sample was not observable by MALDI MS, representing ~300 fmol of analyte in the spot. As 57 fmol was used during the acquisition of the first spectrum, there was still enough analyte present to provide high-quality spectra; however, no peak was seen by MALDI MS analysis, indicating the remaining material is not accessible to MALDI. Unfortunately,

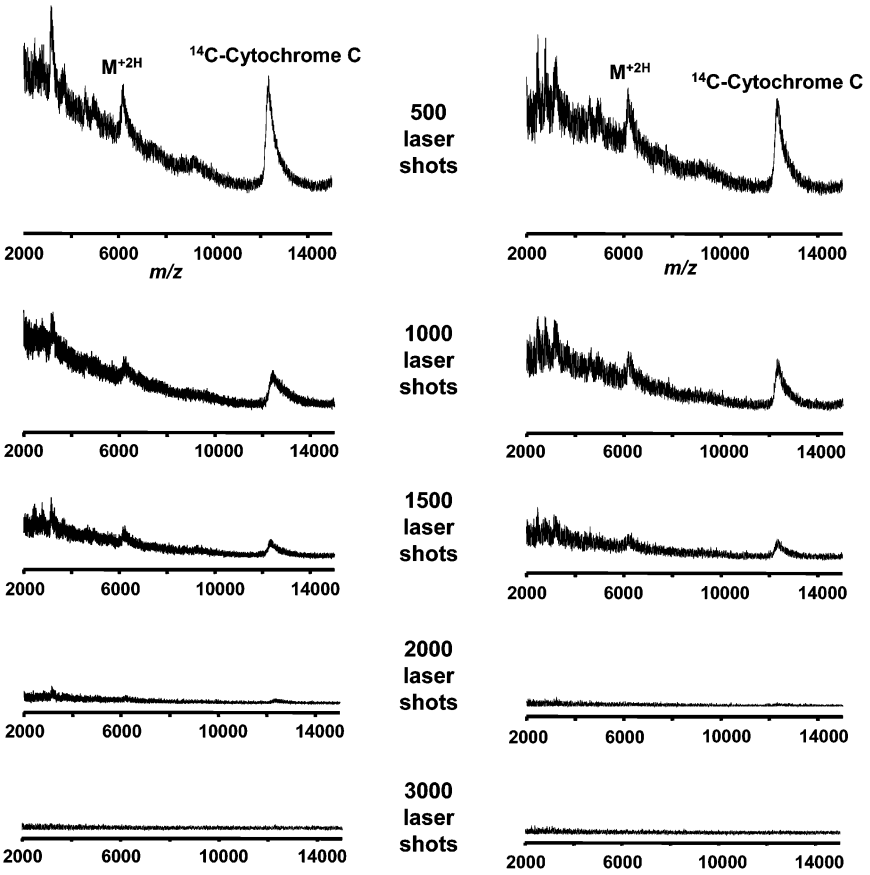

Figure 2. A series of mass spectra from two different sample spots showing the depletion of [^{14}C]-cytochrome *c* by MALDI with the total number of laser shots indicated in the center.

the spatial resolution of the phosphor imager of ~200 μm is not sufficient to determine the spatial distribution of the [^{14}C]-cytochrome *c* within the spot.

A further study was performed to investigate the ~70% of the analyte not MALDI MS observable. Two of the six sample spots from Figure 3 were redissolved in 300 nL of 10 mg/mL DHB. This was done to recrystallize the remaining [^{14}C]-cytochrome *c* with more DHB in order to see whether this would allow more analyte to be desorbed by placing it into fresh crystals, with the larger volume being used to dissolve the samples because of the difficulties of reproducibly adding smaller volumes that would completely dissolve the sample and matrix. The radionuclide results from the spots that were redissolved are shown in Figure 4A. The data before the arrow are the same data found in Figure 3. Two other spots were chosen as controls (Figure 4B), which were not redissolved. Mass spectra were acquired from all four of these spots. No analyte peaks were detected in any of the spots. For the two spots that were redissolved, signal depletion was observed as compared to the controls.

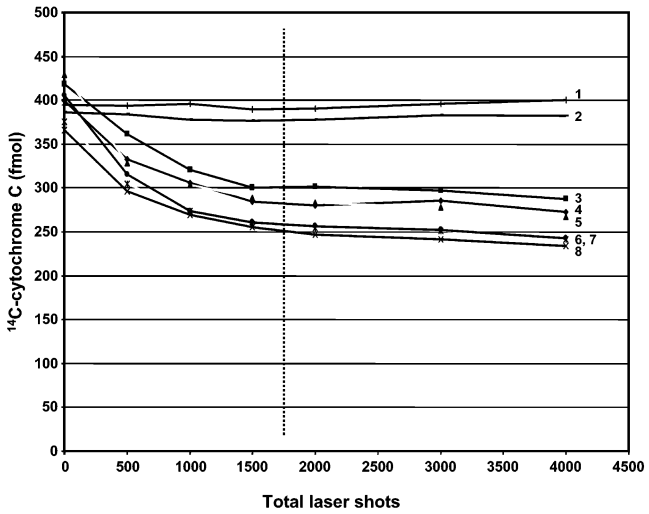

Figure 3. Amount of [^{14}C]-cytochrome *c* in deposited spots detected by a phosphor-imaging detector after a series of mass spectral acquisitions. Six different samples are shown, spots 3−8, to illustrate the consistency of the depletion. The two samples in which no mass spectra were acquired, spots 1 and 2, demonstrate that the depletion is a result of laser desorption/ionization. The vertical dashed line indicates the point in the depletion study that the analyte was no longer detected by MALDI MS.

During the course of several depletion experiments, it was observed that not all the radiation from the previous experiment was removed from the MALDI target after cleaning. Thus, the effectiveness of MALDI target-cleaning methods was tested. Shown in the upper portion of Figure 5 is the radioactivity from deposited DHB spots containing [^{14}C]-cytochrome *c* and ranges from 216 to 384 fmol of analyte in the sample spots. Since the radioactivity comes from the ^{14}C label, this shows the presence and amount of the cytochrome *c*. This target was then cleaned using soapy water and a soft bristled toothbrush followed by a purified water wash. Once the target was dry, the radioactivity from the surface was again detected by a phosphor imager. The resulting image of the radioactivity from the cleaned target is shown on the bottom in Figure 5. The blue indicates no radioactivity while the red show higher levels. From this image

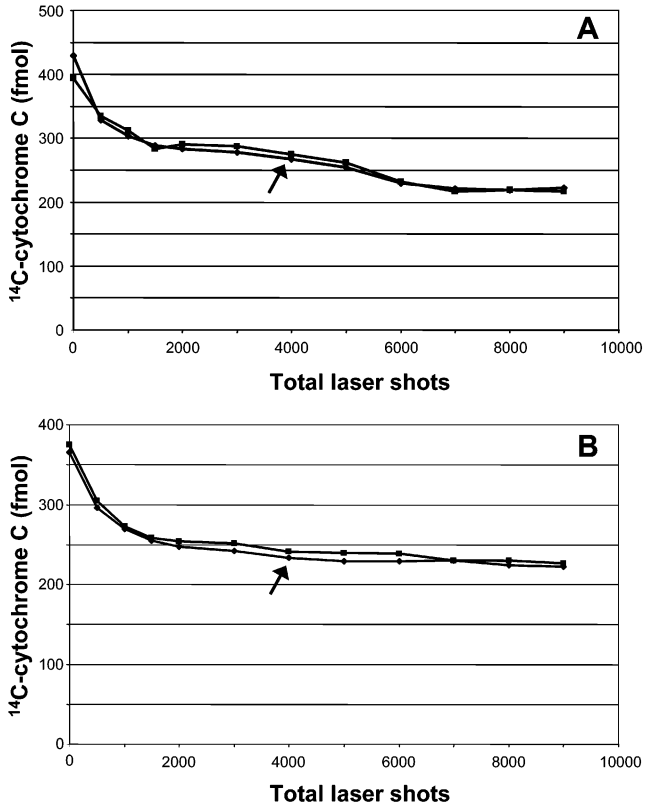

Figure 4. Amounts of [^{14}C]-cytochrome *c* in four spots: (A) two spots that were redissolved in DHB, with the arrow indicating the point additional extra DHB was added; (B) the data from two sample spots (controls) that were not redissolved.

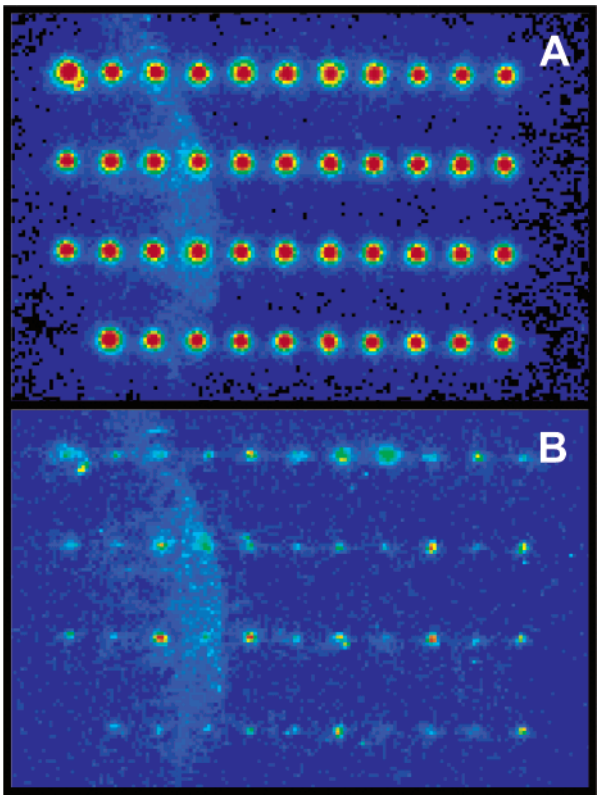

Figure 5. Amount of radioactivity on the MALDI target (A) before and (B) after the target was cleaned. Blue corresponds to low radioactivity, and red, higher levels of radioactivity.

it is observed that analyte is still present, with 3.5−12.4 fmol of cytochrome present in individual spots after cleaning. It is surprising to observe that 2.1 ± 0.9% of the original sample per spot was still left on the target. This suggests that a more aggressive cleaning procedure is required to eliminate contamination between samples. All detectable radioactivity on the target from this study was removed only after boiling the target in a sodium hydroxide solution followed by cleaning of the target in a four-step process with soap, water, ethanol, and acetone.

By performing this depletion study, several pieces of information were obtained: the moles of analyte used during MS acquisition, along with the average amount ablated per laser shot. The depletion from the spots was followed using three different techniques—microscopy, radionuclide detection, and MALDI MS. Only radionuclide detection allows the measurement of the analyte left in a spot after it is no longer observable by MS. As redissolving the analyte in matrix resulted in further analyte desorption, this suggests that much of the [^{14}C]-cytochrome *c* was in a physical state that hinders desorption but that this material can be moved into new matrix crystals that can be further desorbed.

We have shown here that MALDI MS combined with radionuclide detection is a viable analytical technique to gain sensitive and quantitative information on sample consumption during the MALDI process. Additional work is needed to more fully understand the environmental and instrumental factors that caused the [^{14}C]-cytochrome *c* remaining in the spot from not being desorbed. Future work will focus on the role of laser spot size on the desorption process. While quantitative measurements of the material depleted from a MALDI target have not been performed previously, low-attomole samples have been used to generate MALDI spectra,[27,41,42] in agreement with the attomole/laser pulse of desorbed material pulse measured in these experiments. Obviously, the depleted amount should depend on the sample identity, sample spot size, and particular MALDI matrix used. Using the combination of these techniques, we hope to develop optimized methods for small spot preparation and gain information on how different target materials, matrixes, and sample matrix components affect the desorption process.

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