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Technical Report

Use of Single Spot Imaging in MALDI Method Development

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Abstract:

In the process of analytical method development using MALDI, the choice of matrix, ratios of solvents and data acquisition strategies are critical components to achieve optimum signal. Whilst many of the initial stages in method development will relate to the molecular size and chemical nature of the analyte, optimisation of sample preparation and acquisition parameters is necessary to achieve the optimal analytical method for your analyte of interest. MALDI mass spectrometry imaging (MALDI-MSI) is a useful tool to better understand the distribution of analyte to matrix within the spot to assess homogeneity of the preparation. It also provides information to actively determine the optimal acquisition settings (e.g. raster pattern) to achieve both the best signal to noise ratio and reduce spot to spot variation to give more consistent results.

1. Background

In MALDI, a typical method development process begins with assessing spectra to find the optimal matrix. This is usually based on prior experience of similar compounds or extensive literature searches to narrow the field of potential matrices. Following this, attention must be given to the choice of and ratio of organic and aqueous components in both the matrix preparation and the sample, as this is crucial for spot morphology and the distribution of the analyte within the spot. At the same time, it is important to look at instrument parameter settings including laser power, number of shots, pulsed (delayed) extraction values and size and shape of your automated sampling pattern (raster).

Method development is accomplished through repeated analyses with limited alterations to individual conditions for each acquisition to determine the optimal method based on the data acquisition results. However. this provides limited understanding of the effect changes made during sample preparation have on the analyte distribution within the spot.

Single spot MALDI-MSI can provide valuable spatial information to make better informed decisions during method development. By using MALDI-MSI, it is possible to better understand the effects alterations in sample preparation have on spot homogeneity and can also guide sampling (raster) strategies. This ultimately will lead to reduced time and cost for method development and improved data quality.

2. Individual Spot Imaging using MALDI Solutions™ *Data Acquisition*

Fig. 1 shows the co-registration of a standard FlexiMass-SR48 target using the imaging wizard in MALDI Solutions™ *Data Acquisition*. The registrations marks should be coregistered to the centre points of wells A1, A4 and L4. This allows precise selection of individual wells for imaging using the circular raster tool.

All spot images shown were performed at 30 µm spacing. This allows a clear image of the spot composition to be visualised. 50 µm spacing will also produce an adequate image. In addition to spacing, the laser rep rate and the number of shots per profile will impact the run time. Example timings at 200 Hz are shown below (Table 1).

Shots per profile	Approximate Acquisition Times (min:sec)	
	30 µm	50 µm
5:	1:50	1:05
10:	3:46	1:50
50:	20:41	7:49

Table 1 Approximate acquisition times of single well at 30 µm or 50 µm spacing relative to shots per profile

Fig. 1 Coregistration of a FlexiMass-SR48 slide allows areas to be drawn around individual spots precisely for image acquisition

Example 1: Oligonucleotides

3-Hydroxypicolinic acid (3-HPA) is a commonly used matrix for the analysis of oligonucleotides by MALDI. For this spot imaging example, an oligonucleotide sample was prepared using commercially available oligonucleotide standards. The sample contains a 12mer, 20mer, 30mer and 39mer. The sample was desalted and premixed with 3-HPA matrix prior to spotting.

Oligonucleotides in 3-HPA form a characteristic spot with a raised edge and a thin central region. Imaging shows that for all the oligonucleotides, higher distributions are found within the outer raised edge of the sample spot (see Fig. 2). An annular raster strategy for these samples in combination with manual acquisition is likely to yield improved quality spectra. For higher throughput automated acquisitions, implementation of an annular raster strategy in conjunction with a solution that controls the precise location of the spot would be recommended. For example, for accurate spot positioning, samples can be prepared using one of the Shimadzu FlexiFocus™ targets, a single-use target which has been chemically modified to consistently focus the sample in the centre of the well.

Alternatively, a strategy using the data quality option to identify regions which give a higher signal for masses of interest could be implemented.

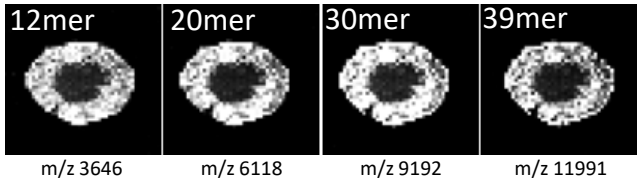

Fig. 2 MALDI MSI spot images of for oligonucleotides in 3-HPA show characteristic doughnut-like distribution.

Using the region of interest (ROI) feature in the *MALDI Solutions*™ IonView™ software, spectra were obtained for a region in the centre and an identically sized region on the outer edge of the spot (See Fig 3). The ROI spectrum of the outer edge showed an increase in signal of approximately 40%.

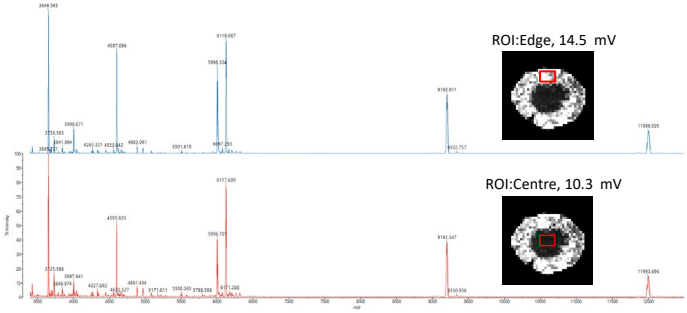

Fig. 3 Region of interest (ROI) spectra taken from the regions highlighted above from the edge and the centre regions of an oligonucleotides sample in 3-HPA show increased signal in the outer regions of the spot.

Whilst the use of 3-HPA is most often recommended for oligonucleotide analysis, groups are still looking into alternative matrices, solvent ratios and additives to improve signal and reduce fragmentation of oligonucleotides. [1] MALDI-MSI spot imaging would be beneficial in these studies for the assessment of spot homogeneity and to optimise data collection strategies to produce more consistent results.

Example 2: Microbial ID Method Development

Whilst bacterial samples are most often prepared using α-Cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid (SA), occasionally the use of 2,5-Dihydroxybenzoic acid (DHB) may be necessary. For example, gram-positive bacteria or other bacteria with particularly strong cell walls may benefit from the softer desorption conditions provided by DHB. In such cases, the matrix helps preserve intact molecular ions, which are crucial for accurate identification. Where a particular matrix is predetermined, spot imaging can be beneficial in establishing the most suitable raster strategy to maximise sensitivity.

For this example, *Escherichia coli* (*E. coli*) DH5α suspension was mixed with 50 mg/mL DHB. The matrix was prepared in a solution containing equal parts of acetonitrile, ethanol and water and a final concentration of 3% TFA. 0.5 µL was spotted onto a FlexiMass-DS sample plate and the spot was allowed to air dry before imaging.

Imaging results showed that the proteins are visible along the length of the matrix crystals formed. During spot drying, DHB co-crystalises with the analyte forming long needle type crystals. These can be fairly heterogenous and lead to 'hot spots'. As can be seen in Fig. 4, the region around the edge of the spot tends to have a higher crystal density. And this can be particularly significant for the less abundant higher mass proteins.

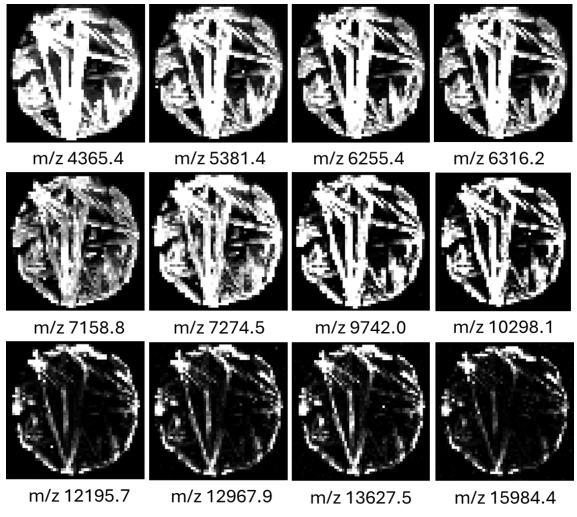

Fig. 4 Images of bacterial proteins where higher m/z (>12000) species show higher abundances at the outer edges of the spot.

In fact, when analysing microbial samples using DHB, an annular raster is part of our preferred acquisition strategy to improve s/n and resolution. Following imaging, *E. coli* DH5α samples were then analysed with either circular or annular rasters using the acquisition parameters shown in table 2. Example spectra are shown in Fig. 5.

Table 2 Manual Analysis Conditions for MALDI-8030

System	: MALDI-8030
Polarity	: Positive
Mass Range	: *m/z* 200-20000
Acquisition	: 20 shots @ 200 Hz
Blanking	: 300
Pulsed Extraction	: 8500
Profiles	: 109

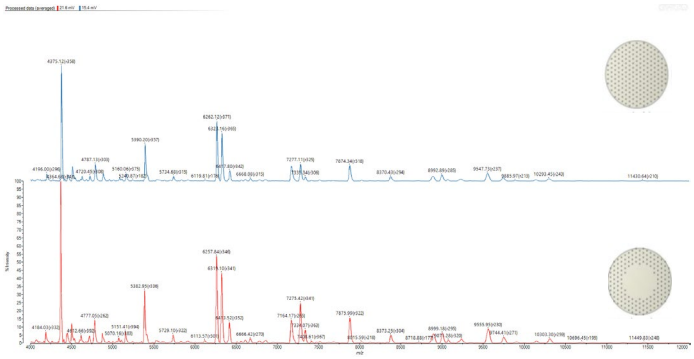

Fig. 5 Processed spectra for manually acquired annular and circular raster strategies of bacterial proteins in DHB showed an approximate 40% increase in signal using an annual raster strategy.

Comparing the processed spectra acquired using either annular or circular raster strategies, an improvement in signal (approx. 40%) was seen with the annular raster strategy across the mass range m/z 2000-20000. This can be attributed to focusing the acquisition on those areas where the crystal coverage is more homogeneous, producing a more consistent high signal.

As previously discussed, use of an annular raster should be combined with either manual acquisition or, for higher throughput, alternative sample spotting strategies to control the location of the spot. Again, an alternative to this strategy would be to use the data quality feature to ensure a minimum level of signal for peaks of interest at each data point acquired.

Comparison of DH5α preparations via spot imaging for DHB with CHCA (see Fig. 6) or SA (see Fig. 7) show that for the majority of proteins, both CHCA and SA show more homogeneous spots which would not require use of an annular raster. However, with lower abundance and increasing m/z ratios a trend is seen which suggests an annular raster strategy may in some circumstances be beneficial. For example, studies investigating higher mass proteins (> m/z 10,000), may find adoption of an annular raster strategy would improve the overall quality of spectra.

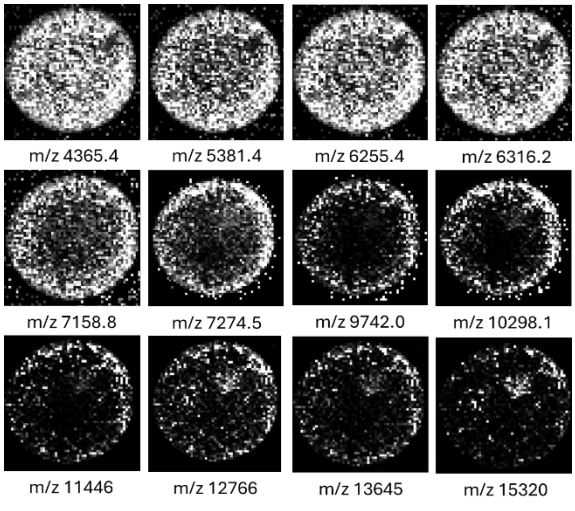

Fig. 6 With CHCA, images of bacterial proteins show higher intensities are observed at the outer edges of the spot. This becomes more noticeable at higher m/z.

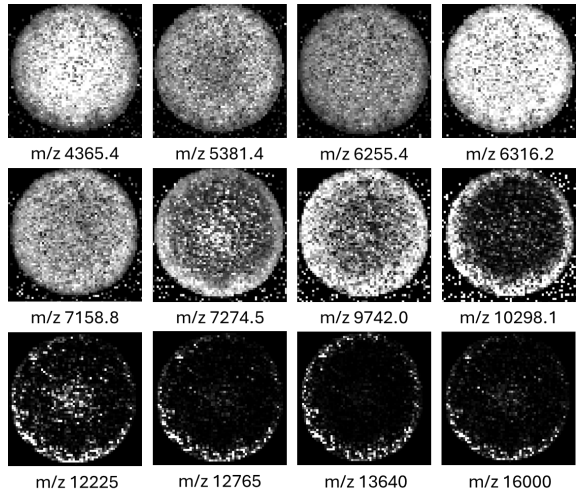

Fig. 7 With SA, images of bacterial proteins show higher intensities are observed at the outer edges of the spot. This becomes more noticeable at higher m/z.

A possible explanation for this trend could be that as the spot dries, the matrix:sample ratio becomes more optimal for these lower abundance, higher mass peaks, resulting in an increase in signal intensity.

Other strategies to ensure optimal signal include utilising the data quality function within MALDI Solutions *Data Acquisition*. Data quality is used in conjunction with an automated acquisition strategy to define the criteria by which profiles are summed into the final acquired spectrum. Data quality allows the selection of advanced acquisition strategies based on these criteria. Another alternative would be to disrupt the crystal formation during spotting which results in a more uniform distribution albeit with a reduction in resolution, although with average masses used for bacterial acquisitions this is of less significance (See Fig. 8).

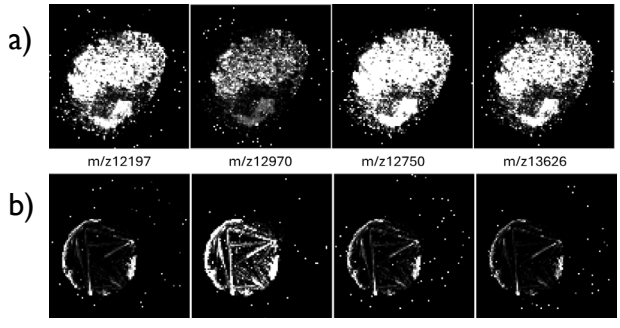

Fig. 8 Disruption of DHB crystal formation (row a) during spotting gives rise to a more homogenous distribution of bacterial proteins than is seen in standard sample spotting procedures (row b).

Example 3: Proteins & Peptides

The analysis of proteins and peptides is well established using MALDI-TOF. A variety of matrices and preparation methods have been developed. Figure 9 and Figure 10 show an example protein (bovine serum albumin, BSA) and an example peptide (Glu-1-fibrinopeptide, Glu-1) prepared using SA or CHCA as matrix respectively. Each spot shows a different preparation technique. All show good homogeneity albeit with some variation in intensity throughout the spot. It can also be seen that there are varying levels of intensity in general between the different preparation techniques.

For BSA, the following spotting techniques were used:

a) 0.5 µL BSA sample was spotted followed by 0.5 µL of SA (20 mg/mL in 50/50 Acetonitrile/0.1% TFA).
b) Prior to spotting, the sample well was precoated with 20 mg/mL SA in 50/50 acetonitrile/0.1% TFA. 0.5 µL BSA sample was spotted followed by 0.5 µL of SA (20 mg/mL in 50/50 Acetonitrile/0.1% TFA)
c) Prior to spotting, the sample well was precoated with 20 mg/mL SA in acetone. 0.5 µL BSA sample was spotted followed by 0.5 µL of SA (20 mg/mL in 50/50 Acetonitrile/0.1% TFA)

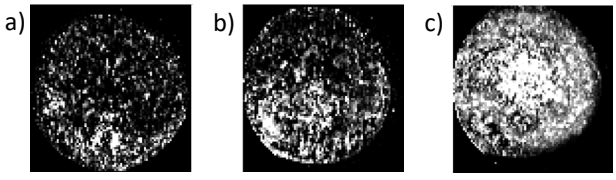

Fig. 9 MALDI-MSI spot images of BSA in SA using a variety of spotting techniques.

For Glu-1-fibrinopeptide, the following spotting techniques were used:

a) 0.5 µL Glu-1 sample was spotted followed by 0.5 µL of CHCA (5 mg/mL in 50/50 Acetonitrile/0.1% TFA).
b) Prior to spotting, the sample well was precoated with 5 mg/mL CHCA in 50/50 acetonitrile/0.1% TFA. 0.5 µL Glu-1 sample was spotted followed by 0.5 µL of CHCA (5 mg/mL in 50/50 Acetonitrile/0.1% TFA)

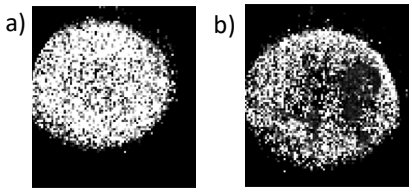

Fig. 10 MALDI-MSI spot images of Glu-1 in CHCA using a variety of spotting techniques.

The final example shows MALDI-MSI of a 7-peptide calibration mixture (See Fig. 11). 0.5 µL calibration sample was spotted followed by 0.5 µL of CHCA (5 mg/mL in 50/50 Acetonitrile/0.1% TFA). As can be seen in Fig. 11, differing distributions and intensities are observed for the different peptides. The most striking difference is the distribution of the N-acetyl-renin substrate at m/z 1801. It is possible that this particular peptide is more hydrophobic than the other peptides in the mixture. This serves as a reminder to us that not all molecules within a class will respond similarly to any given matrix or preparation technique. Particularly when using internal standards, close attention should be paid to ensuring similar spatial distributions are achievable or otherwise compensated for through acquisition strategies.

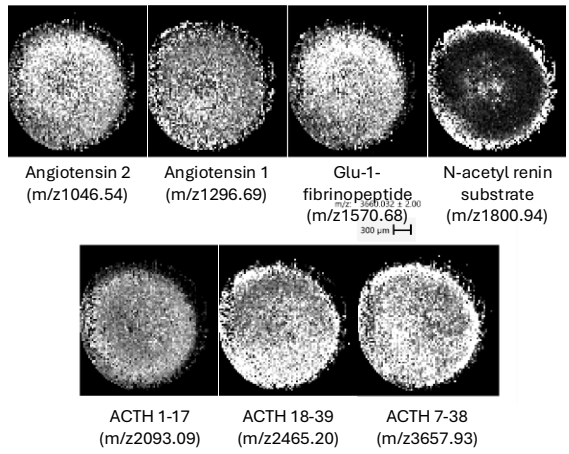

Fig. 11 MALDI-MSI spot images of individual peptides in a 7-peptide calibration mix spotted with CHCA.

Conclusions

The use of MALDI-MSI during method development can provide valuable spatial information allowing the analyst to make informed decisions in the optimization of acquisition parameter settings.

The ratios of solvents both within and between matrix and sample can significantly alter spot morphology and the distribution of the analytes throughout the spot. Initial work is often assessed by spot morphology and analyte sensitivity. This allows fast assessment of a number of different sample preparation conditions. MALDI-MSI can facilitate this to give a better understanding of how the analyte distribution is changing within the sample and allows for fine tuning of optimal spot homogeneity, taking into consideration effects such as analyte affinity to solvents and drying times of preparations that will also affect the spatial distribution of the analyte of interest.

Where the choice of matrix may be limited and homogenous analyte distribution cannot be achieved, altering acquisition strategies can improve both signal to noise and resolution of your spectra. MALDI-MSI provides a quick, simple way to gain relevant information to choose an optimal raster approach, and this simple alteration can lead to significant improvements in spectrum quality.

Alternative strategies can also be utilized alongside the optimal sample preparation to provide consistently higher quality results. The use of MALDI-MSI provides greater insight into the process allowing for focused, reasoned method development.

References

1. Dietrich *et al*, Rapid Communications in Mass Spectrometry, 2025; 39:e10061

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