Normalization techniques for high-throughput screening by infrared matrix-assisted laser desorption electrospray ionization mass spectrometry

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Funding information
National Institute of General Medical Sciences; NIH, Grant/Award Number: R01GM087764; AbbVie, Grant/Award Number: None

Abstract
Mass spectrometry (MS) is an effective analytical tool for high-throughput screening (HTS) in the drug discovery field. Infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) MS is a high-throughput platform that has achieved analysis times of sub-seconds-per-sample. Due to the high-throughput analysis speed, methods are needed to increase the analyte signal while decreasing the variability in IR-MALDESI-MS analyses to improve data quality and reduce false-positive hits. The Z-factor is used as a statistic of assay quality that can be improved by reducing the variation of target ion abundances or increasing signal. Herein we report optimal solvent compositions for increasing measured analyte abundances with direct analysis by IR-MALDESI-MS. We also evaluate normalization strategies, such as adding a normalization standard that is similar or dissimilar in structure to the model target drug, to reduce the variability of measured analyte abundances with direct analyses by IR-MALDESI-MS in both positive and negative ionization modes.

Keywords
high-throughput screening, IR-MALDESI, normalization, Orbitrap mass spectrometer

1 | INTRODUCTION

Novel drug discovery technologies have made it possible to rapidly produce thousands of potential new drugs that require rapid and accurate screening.¹ High-throughput screening (HTS) methods are therefore essential to produce drug hits within a feasible amount of time. Mass spectrometry (MS) is an analytical technique that can accurately detect hundreds to thousands of molecules simultaneously, making it an effective method to efficiently screen drug hits. Liquid chromatography (LC) is frequently coupled with MS for screening diverse biochemical assays with high accuracy and sensitivity, but throughput is limited due to the additional time required for separation before mass analysis.²-⁴ Matrix-assisted laser desorption/ionization (MALDI)-MS is another popular analytical tool that can achieve a sub-second per sample analysis rate.⁵-⁷ However, MALDI-MS requires the addition of an organic matrix before analysis, increasing sample prep time and background signal.⁸ Typically, direct analysis platforms are favored for HTS due to the limited sample preparation required before analysis. The first direct analysis platforms were desorption electrospray ionization (DESI) and direct analysis in real
Infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) is a hybrid, ambient ionization source that combines the benefits of electrospray ionization (ESI) and MALDI. IR-MALDESI is commonly used for MS imaging applications but has also been characterized as a high-throughput, direct analysis platform. The IR-MALDESI source has been extensively described previously. We also tested the normalization to a polymer internal standard. Finally, we determined the effect of the Orbitrap Exploris 240 mass spectrometer’s ion optics on the variability of target ion abundances. Our evaluation was based on measuring the variability of the various oligomer abundances to measure the variability change over a wide m/z range.

2 | EXPERIMENTAL

2.1 | Materials

LC-MS-Grade water, methanol (MeOH), acetonitrile (ACN), formic acid (FA), and acetic acid (HOAc) for the electrospray solvent were purchased from Fisher Scientific (Nazareth, PA, USA). Caffeine was also purchased from Fisher Scientific (Hampton, NH, USA). Theophylline, riboflavin, glutathione (GSH), benzoic acid, sorbic acid, MRFA (peptide), and polyethylene glycol (PEG) were purchased from Sigma Aldrich (St. Louis, MO, USA). Homo-glutathione (hGSH) was purchased from Bachem (Bubendorf, Switzerland). Stable isotope-labeled glutathione (SiL-GSH, $^{13}$C$_2$N) and stable isotope-labeled caffeine (SiL-caffeine, $^{13}$C$_3$) were purchased from Cambridge Isotope Laboratories, Inc (Tewksbury, MA, USA). Splash Lipidomix Mass Spec Standard (Splashmix) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). Well plates (100 µl) were purchased from Brand GMBH & CO KG (Wertheim, Germany).

2.2 | IR-MALDESI-MS platform

The IR-MALDESI source has been extensively described previously. The IR-MALDESI source is currently coupled to an Orbitrap Exploris 240 mass spectrometer (Exploris 240, Thermo Fisher Scientific, Bremen, Germany). Electronic triggering of the Exploris 240, JGMA laser, and sample stage position were all controlled using RastiX, an in-house software paired with an Arduino microcontroller. A single burst of nine pulses (1.2 mJ/burst) from the JGMA 2970-nm laser (Burlington, MA, USA) focused on the meniscus of the sample, which desorbed neutrals from the sample well plate. The orthogonal ESI plume was created by applying a voltage of 3.6 kV at the 50-µm ID emitter tip and a fixed flowrate of 2 µl/min. The ESI solvent composition varied between experiments. The automatic gain control (AGC) of the Exploris 240 was disabled and the injection time was fixed at 15 ms. Mass spectra were collected over m/z 175–900 in positive mode for the solvent composition optimization experiments. Mass spectra were recorded over m/z 100–350 in negative mode and m/z 175–450 in positive mode for the normalization method evaluation. For the PEG 600 normalization method, mass spectra were recorded in positive mode over m/z 175–1000. The internal lock mass (fluoranthenes, m/z 202.0777) was enabled to obtain parts-per-million mass measurement accuracy. The mass spectrometer resolution was fixed at 240,000 at m/z 200 and the S-lens RF-level was fixed at 70% for all experiments.

\[ Z’ = 1 - \frac{3\sigma_+ + \sigma_-}{\mu_+ - \mu_-} \]
2.3 | Optimizing sample solvent composition

To determine the optimal sample composition, 100-μM caffeine, and 10-μL Splash Lipidomix (Splashmix) were mixed with 190 μL of varying solvent compositions of water with methanol (methanol series) or water with acetonitrile (acetonitrile series) to make a 200-μL sample. The methanol series covered compositions 5%-80% methanol (MeOH) /95%-20% H2O (v/v) and the acetonitrile series covered 0%-75% acetonitrile (ACN)/100%-25% H2O (v/v). The electrospray solution composition for the methanol series consisted of 0.2% FA in 50/50% MeOH/H2O (v/v) and the electrospray solution for the acetonitrile series consisted of 0.2% FA in 60/40% ACN/H2O. Both solvent compositions were previously found to be optimal for lipidomic IR-MALDESI-MSI analyses. The abundances of each lipid were normalized to a similar and dissimilar normalization standard respectively. Also, the %RSD of raw GSH abundances was compared to the %RSD of GSH abundances normalized to the TIC, where yi is the abundance of the ith peak in the spectrum. The normalization techniques were then repeated in positive ionization mode with caffeine as the model drug, theophylline as the similar normalization standard, and riboflavin as the dissimilar standard.

2.6 | Measuring variability as a function of m/z

A solution of 1-μM MRFA and 3.0-μg/ml PEG in 10/90% MeOH/H2O (v/v) was used to measure the variation of detected ions based on the ion’s m/z due to the Exploris 240 ion optics. Also, the effectiveness of a polymer normalization standard was also studied to determine if differences in m/z between a target ion and the normalization standard affected the reduction in variability. Normalizing to a polymer was also studied to determine if the measured variability decreases depend on the difference in abundance between the target ion and the normalization standard.

2.4 | Sample preparation for evaluating normalization techniques (negative and positive mode)

For negative mode, a solution composed of 100-μM GSH, hGSH, benzoic acid, and sorbic acid in 10/90% MeOH/H2O (v/v) modeled a drug discovery sample. The ESI solution consisted of 1-mM HOAc and 1-μM SIL-GSH in 50/50% MeOH/H2O. The SIL-GSH was used to monitor the ESI stability during the analysis. GSH modeled a target molecule while hGSH and benzoic acid acted as similar and dissimilar normalization standards, respectively.

For positive mode, a solution of 100-μM caffeine, theophylline, and riboflavin in 10/90% MeOH/H2O (v/v) was used to model a drug discovery sample. The ESI composition was 0.2% FA and 1-μM SIL-caffeine in 50/50% MeOH/H2O. SIL-caffeine was used to monitor the stability of the ESI during the analysis. Caffeine was the model target molecule, theophylline was used as a similar normalization standard, and riboflavin was used as a dissimilar normalization standard.

2.5 | Evaluating assay variation and normalization techniques

The relative standard deviation (%RSD),

\[
\% \text{RSD} = \frac{\sigma_A}{\mu_A} \times 100\%
\]

was chosen to measure target ion variation, where \(\sigma_A\) is the standard deviation of the peak A abundances and \(\mu_A\) is the mean of peak A abundances. In negative mode, %RSD of GSH abundances was compared to the %RSD of GSH normalized to hGSH and GSH normalized to benzoic acid to measure the decrease in variation of GSH normalized to a similar and dissimilar normalization standard respectively. Also, the %RSD of raw GSH abundances was compared to the %RSD of GSH abundances normalized to the TIC, where yi is the abundance of the ith peak in the spectrum. The normalization techniques were then repeated in positive ionization mode with caffeine as the model drug, theophylline as the similar normalization standard, and riboflavin as the dissimilar standard.

3 | RESULTS AND DISCUSSION

3.1 | Sample solvent composition optimization

The sample solvent composition was optimized to increase the abundances of target ions for an IR-MALDESI-MSI analysis. The 100-μM caffeine [M+H]+ (m/z 195.0877) was mixed with 10 μL of Splashmix in various compositions of water mixed with either methanol or acetonitrile. The methanol compositions tested were 5%-80% MeOH while the acetonitrile compositions tested consisted of 0%-75% ACN. Splashmix lipid abundances were summed together (sum-of-lipids) and compared to the caffeine abundances across all solvent compositions to maximize total target abundances. These abundances were
also averaged for comparison to understand the overall effects of solvent composition. These comparisons are shown in Figure 1.

The averaged abundances were comparable (∼1.5 × 10^7 charges/s) across all compositions tested. Since most biochemical samples solvents are composed entirely of water, low organic compositions were preferred to ideally model real samples. Therefore, the optimal aqueous solvent composition was 5% MeOH and 0% ACN for the methanol series and acetonitrile series respectively. If a higher organic phase is needed, 40% MeOH or 45% ACN solvent composition would also be acceptable. Interestingly, we found that each Splashmix lysophosphatidylcholine (LPC) abundance increased by an order of magnitude for the 15% ACN composition likely due to an extraction effect on the LPCs facilitating easier desorption of neutrals. The increase in LPC abundances resulted in increased sum-of-lipid abundances, but since caffeine abundances did not increase for 15% ACN composition, this was not considered the true optimal composition. However, this shows the 15% ACN composition should be used for lipid direct analyses by IR-MALDESI-MS. The %RSD of measured caffeine and sum-of-lipids abundances for each set of 100 scans over each composition was used as a measure of variability within each experiment and is shown in Figure S1. The 40% MeOH composition showed a lower calculated variability compared to the 5% MeOH composition. Though the repeatability of the measured lipid abundances was better for the 20% MeOH composition, the repeatability of the measured caffeine abundances and the variability for both analyses were better for the 5% MeOH condition. Since the abundances measured for the sum-of-lipids from the 5% MeOH composition were greater than the 20% MeOH and the purpose of this study was to increase analyte ion abundances, the 5% MeOH composition was chosen as optimal. While an optimal composition is stated at 5% MeOH and 0% ACN, the study showed that IR-MALDESI tolerates a wide range of sample solvent compositions.

3.2 | Evaluation of normalization techniques (negative mode)

A sample of 100-μM GSH [M-H]^− (m/z 306.0765), hGSH [M-H]^− (m/z 320.0922), benzoic acid [M-H]^− (m/z 121.0295), and sorbic acid [M-H]^− (m/z 111.0452) in 10/90% MeOH/H2O was analyzed by IR-MALDESI-MS. The addition of 10% MeOH solvent composition was used to model the addition of a small amount of organic phase from a stock solution. The electrospray solvent was 1-mM HOAc and 1-μM SIL-GSH in 50/50% MeOH/H2O. The SIL-GSH [M-H]^− (m/z 309.0803) was used to monitor the stability of the electrospray during analysis. Extracted ion chronograms of each species present in the sample are shown in Figure 2A. The chemical structure of each analyte is shown in Figure 2B.

Benzoic acid, sorbic acid, GSH, and hGSH were sampled from the well plate and their abundances are functions of both the laser desorption event and then electrospray ionization. The chronograms of benzoic acid and sorbic acid were nearly identical. GSH and hGSH chronograms were also identical but were dissimilar to the chronograms of benzoic acid and sorbic acid. The TIC was visibly more similar to the chronograms of benzoic acid and sorbic acid likely because these species were the largest abundances in the spectra and therefore contributed to a large portion of the TIC. This is shown in the mass spectrum in Figure S2. The chronogram of SIL-GSH is visibly different from the all other chronograms and was expected because SIL-GSH abundances are only a function of ESI and not the laser desorption event.

The abundances and %RSD of GSH and benzoic acid measured over 100 scans are shown in Figure 3A and Figure 3B, respectively. The %RSD of unnormalized GSH was 47.4%. When normalized to hGSH, the %RSD significantly decreased to 5.5%, Figure 3C. Similarly, when benzoic acid was normalized to sorbic acid, a similar compound,
FIGURE 2  (A) Chronograms of the TIC and each analyte of interest. The 100-μM sorbic acid, benzoic acid, GSH, and hGSH were sampled out of 100-μl wells. The 1-M SIL-GSH was infused in the electrospray to monitor the electrospray variability during the analysis. The popout boxes show similarities in the chronograms from species sampled from the well plates and show the relative stability of the electrospray ionization (ESI) solution shown by the orthogonality of the SIL-GSH chronogram. (B) The structure of each molecule of interest. hGSH was used as a similar normalization standard to GSH while sorbic acid and benzoic acid were used as dissimilar normalization standards for GSH. The asterisks (*) represent either 15N or 13C based on the atom it indicates.

FIGURE 3  Abundances of (A) GSH (m/z 306.0765), and (B) benzoic acid (m/z 121.0295) measured over 100 scans. Abundances of (C) GSH normalized to hGSH (m/z 320.0922), and (D) benzoic acid normalized to sorbic acid (m/z 111.0452). The black line indicates the average unnormalized abundance or normalized abundance for each species over 100 scans. The corresponding %RSD and its decrease are displayed above each plot (n = 100).
the %RSD (21.8%) decreased to 7.7% (Figure 3D). This shows that similar compounds are effective normalization standards to reduce variation for direct analyses by IR-MALDESI-MS in negative mode.

To test if dissimilar compounds are effective normalization standards, GSH was then normalized to benzoic acid and sorbic acid. Abundances of 100-μM benzoic acid were significantly greater than all other ion signals in the spectra so the concentration was reduced to 10 μM to minimize potential signal suppression of the other target ions. The raw GSH abundances, GSH abundances normalized to sorbic acid, GSH normalized to benzoic acid, and GSH normalized to the TIC are shown in Figure 4. The average mass spectrum of the negative mode variability sample is shown in Figure S3.

The %RSD of GSH normalized to sorbic acid (35.7%) and normalized to benzoic acid (34.8%) were lower compared to the unnormalized %RSD (39.1%). However, the decrease in variation from normalizing to a dissimilar compound as shown previously (Figure 3C,D). Finally, normalizing to the TIC reduced the %RSD of GSH from 39.1% to 30.1% and resulted in a larger variability decrease compared to normalizing to a dissimilar compound (Figure 4D).

### 3.3 Evaluation of normalization techniques (positive mode)

The normalization methods were restudied in positive ionization mode with 100-μM caffeine [M + H]^+ (m/z 195.0877), theophylline [M + H]^+ (m/z 181.0720), and riboflavin [M + H]^+ (m/z 377.1456) in 10/90% MeOH/H2O used as a model HTS sample to determine if normalization to theophylline (similar) and riboflavin (dissimilar) can reduce the variation of caffeine abundances. The electrospray consisted of 50/50% MeOH/H2O with 0.2% FA and 1-μM SIL-caffeine [M + H]^+ (m/z 198.0977) to monitor the stability of the electrospray during an analysis. Chronograms of each species present in the model sample are shown in Figure 5A with their respective chemical structures shown in Figure 5B.

Caffeine, riboflavin, theophylline, and the TIC abundances were all functions of the laser desorption event and the electrospray ionization of the IR-MALDESI-MS analysis. All four chronograms were identical to each other. The measured SIL-caffeine abundances were only a function of the ESI of the IR-MALDESI-MS analysis so the resulting chronogram was not similar to the analytes sampled from the well plate.

Figure 6A-D show the raw caffeine abundances, caffeine abundances normalized to theophylline, riboflavin, and the TIC, respectively. The average mass spectrum of the positive mode variability sample is shown in Figure S4. The %RSD significantly decreased from 18.4% to 7.4% when normalized to the similar theophylline. The %RSD also decreased to 12.7% when normalized to the dissimilar riboflavin compound, but the reduction is not as significant as normalizing to a similar compound, similar to the negative mode data. Analogous to the observation made in the negative mode, the variation reduction normalized to the TIC (%RSD = 7.5%) was greater than normalizing to a dissimilar compound, and comparable to normalizing to a similar compound. This is likely because the analytes of interest are the most abundant species in each mass spectrum. As discussed for negative mode, normalizing to a similar compound provided the greatest...
FIGURE 5  (A) Chronograms of the total ion current (TIC) and each analyte of interest. The 100 μM of riboflavin, caffeine, and theophylline were sampled out of 100-μl well plates. The 1-μM SIL-caffeine was added to the electrospray solvent to monitor the stability. The popout boxes show similarities in the chronograms from species sampled from the well plates and to show the relative stability of the electrospray ionization (ESI) shown by the orthogonality of the SIL-caffeine chronogram. (B) The structure of each molecule of interest. Theophylline was used as a similar normalization standard to caffeine and riboflavin was used as a dissimilar normalization standard. The asterisks (*) represent 13C in SIL-caffeine.

FIGURE 6  (A) Caffeine (m/z 195.0877) abundances measured over 100 scans. Caffeine abundances normalized to (B) theophylline (m/z 181.0720), (C) riboflavin (m/z 377.1456), and the (D) total ion current (TIC). The abundances of SIL-caffeine (m/z 193.0977) were subtracted from the TIC abundances before normalization. The black line indicates the average unnormalized abundance or normalized abundance for each analysis over 100 scans. The corresponding %RSD and its decrease are displayed above each respective plot (n = 100).
The unnormalized %RSD (circles) of each PEG polyethylene glycol in 10/90% MeOH/H₂O was analyzed by IR-MAL-dances based on their To determine if the ion optics affect the variability of target ion abundances, the %RSD of MRFA single (blue) and doubly (red) charge state ions of MRFA (m/z 524.2650, m/z 262.6361) to the most abundant PEG oligomers resulted in comparable normalized %RSD values. This shows that differences in m/z do not affect the variability decrease provided by normalization. The average mass spectrum of the MRFA and PEG oligomer sample is shown in Figure S5.

To determine if normalization is affected by the abundance of the normalization standard, the %RSD of normalized MRFA was plotted against the difference in abundance (ΔAbundance) between the PEG oligomer and both MRFA charge states (1+ and 2+) (Figure 7B).

\[ \Delta \text{Abundance} = A_{\text{PEG}} - A_{\text{MRFA}} \]

where Δabundance is the difference in abundance, \( A_{\text{PEG}} \) is the abundance of the PEG oligomer, and \( A_{\text{MRFA}} \) is the abundance of MRFA. For all three replicates, normalizing MRFA abundances to the most abundant PEG oligomers decreased the %RSD significantly. This means normalizing to the most abundant PEG oligomer peaks is an effective method for reducing variability in an IR-MALDESI-MS direct analysis.

For less abundant PEG oligomers, the normalized variability increased relative to normalizing to the most abundant PEG peak. A polymer standard can be added to a HTS assay if needed, and the target ion abundance can be normalized to the most abundant polymer oligomer peak to reduce the variability of an analysis.

4 | CONCLUSIONS

We found the optimal solvent compositions for achieving maximized target ion abundances with IR-MALDESI-MS direct analysis were 5/95% MeOH/H₂O and 0/100% ACN/H₂O, respectively. IR-MALDESI can tolerate a wide range of sample solution compositions of MeOH/H₂O or ACN/H₂O mixtures. Two model biochemical samples were analyzed to determine if similar and dissimilar species could be used as internal standards to reduce abundance variation in HTS analyses by IR-MALDESI-MS for positive and negative mode. For both polarity modes, normalizing to a similar standard showed the best performance in reduction of analysis variability, followed by normalization to the TIC, and the last is normalization to a dissimilar compound. The ion optics of the mass spectrometer did not significantly increase variability with respect to an ion’s m/z. Finally, PEG 600 polymer can be spiked into a sample as a versatile normalization standard due to its wide m/z range of oligomer peaks for which normalizing to the most abundant oligomer peak resulted in a significant reduction in target ion abundance variation.

ACKNOWLEDGEMENTS

All mass spectrometry measurements were conducted in the Molecular Education, Technology and Research Innovation Center (METRIC) at North Carolina State University. Funding for this...
study was provided in part by AbbVie and a grant from the NIH (R01GM087964).

CONFLICT OF INTEREST
The authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT
Data will be made available on METASPACE or other suitable resource once accepted for publication.

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SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

How to cite this article: Knizner KT, Bagley MC, Pu F, Elsen NL, Williams JD, Muddiman DC. Normalization techniques for high-throughput screening by infrared matrix-assisted laser desorption electrospray ionization mass spectrometry. J Mass Spectrom. 2022;57(6):e4869. doi:10.1002/jms.4869