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Mass recalibration of FT-ICR mass spectrometry imaging data using the average frequency shift of ambient ions

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Abstract

Achieving and maintaining high mass measurement accuracy (MMA) throughout a mass spectrometry imaging (MSI) experiment is vital to the identification of the observed ions. However, when using FTMS instruments, fluctuations in the total ion abundance at each pixel due to inherent biological variation in the tissue section can introduce space charge effects that systematically shift the observed mass. Herein we apply a recalibration based on the observed cyclotron frequency shift of ions found in the ambient laboratory environment, polydimethylcyclsiloxanes (PDMS). This calibration method is capable of achieving part per billion (ppb) mass accuracy with relatively high precision for an infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) MSI dataset. Comparisons with previously published mass calibration approaches are also presented.

INTRODUCTION

The ability to obtain high resolving power (RP) mass spectra with high mass measurement accuracy (MMA) is one of the key advantages of Fourier transform mass spectrometry (FTMS).[1-2] When applied to complex sample analysis these benefits of FTMS cannot be understated.[3] High RP allows for the resolution of ions that are closely arranged in m/z space. This effective increase in the mass spectral peak capacity is of the utmost importance in the analysis of complex mixtures, specifically the molecular imaging analysis of biological tissue sections where prior chromatographic separations are not typically feasible. [4-6] While high RP may not be innately required to obtain high mass measurement accuracy, the resolution of isobaric species is a prerequisite to accurate mass measurement. In addition, the ability to make highly accurate mass measurements can greatly reduce the number of possible elemental compositions that map to a particular mass thereby increasing the confidence of ion identification.[7-11]

A linear and m/z -independent systematic shift in the cyclotron frequency with increasing ion populations was observed in the early ion cyclotron resonance (ICR) and Fourier transform ICR (FT-ICR) literature and was mainly attributed to the effects of Coulombic space charge. [12-15] Similar space charge effects have also been shown to influence the axial frequency in Orbitrap mass spectrometers.[16-18] Thus, the ability to control the magnitude of the ion population from scan to scan is vital for maintaining accurate mass measurements with external calibration throughout an experiment in both FT-ICR and Orbitrap mass spectrometers. One method for achieving ion population control in hybrid FTMS instruments is to externally trap the ions and utilize automatic gain control (AGC).[19-21] For some instruments, AGC is a short prescan event that is used to essentially determine the

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rate at which ions are being generated by measuring the ion current obtained from the mass range of interest for a given period of time. For continuous ionization sources, such as electrospray ionization (ESI), the length of time that the ions are accumulated in the subsequent analytical scan is adjusted in order to reach a target number of ions (AGC target) based on the ionization rate determined during the prescan event.[22] For pulsed ionization sources, like matrix-assisted laser desorption ionization (MALDI), the prescan event determines the number of ions generated per laser pulse. The number of pulses required to reach the AGC target is then varied for the analytical scan.[23-24] This hardware control over the magnitude of the ion population from spectrum to spectrum allows for the use of an external calibration that is obtained at the same ion density. However, despite the incorporation of such controls, there are a few applications, including molecular imaging, where varying the accumulation time or number of laser pulses from scan to scan may not be desired or applicable. One such example is the case of IR laser ablation where the penetration depth and amount of material removed per pulse is significantly large, thereby limiting the number of allowable pulses in one position. [25]

Aside from implementing hardware controls, a number of researchers have also investigated various calibration techniques to combat the effects of space charge and maintain high mass accuracy. Conversion of the observed cyclotron frequency to m/z in FT-ICR is typically accomplished through the two-parameter calibration function proposed by either Franci (Equation 1) or Ledford (Equation 2).[26-27] These two equations have been shown to provide very similar results[28] and they serve as the framework for most of the proposed FT-ICR re-calibration techniques.

$$\frac{m}{z} = \frac{A}{f+B} \quad 1$$

$$\frac{m}{z} = \frac{A}{f} + \frac{B}{f^2} \quad 2$$

In both of these equations the A term is related to the magnetic field strength and the B term accounts for the magnetron motion as well as the electric field generated primarily from the trapping potentials with some contribution from the ion population itself. Wang et al. were able to improve MMA through the inclusion of two more terms and a C parameter to the Ledford calibration.[29] The third term accounted for up to 20 ppm of the observed mass error whereas the fourth term was found to be relatively negligible with only a sub-ppm contribution. A few others have observed MMA improvements by including a third term to account for the influence of the individual ion abundance.[30-36] The basis for the addition of this third term is founded on the notion that ions of the same m/z will only influence and feel influence from ions of different m/z , i.e. an ion cloud of one m/z won't space charge itself.[37-39] Despite there being some experimental evidence that suggests this assumption may not be entirely valid,[26, 40] recent simulations identified these shifts as relating to image charge interactions rather than space charge effects.[41] Given that a majority of the observed shift in cyclotron frequency is linearly dependent on the total ion population, other calibration methods include a term that relates to the total ion abundance allowing for MMA in the low ppm range.[33-36, 42-43] While some of the external calibration methods are capable of achieving mass accuracies within 5 ppm, it has been demonstrated that internal calibration can provide significant improvement and in some cases reach the ppb range. [44-45] These internal calibrants can be mixed with the sample or introduced from a secondary ion source[46-54]; however, it is also possible to utilize constituents of the sample[55-61] or ambient PDMS[62] ions as calibrants.[42, 57-58, 63]

There have also been a few calibration methods introduced for the Orbitrap that correct for systematic shifts in axial frequency. The simplified conversion from axial frequency to m/z for the Orbitrap is based on a single parameter equation (Equation 3).[64]

$$\frac{m}{z} = \frac{A}{f^3}$$

Olsen et al. used an ambient PDMS ion as a lock mass to re-calculate a new A parameter for each scan to account for systematic frequency shifts.[63] This lock mass calibration has been incorporated into the software for Orbitrap based instrumentation and was capable of maintaining most of the identified ions within 1 ppm. Because of the origin of these PDMS ions (volatile compounds from deodorants and shampoos), their abundances can vary significantly. To stabilize the signal from the PDMS ions throughout an LC-MS run, Lee and coworkers simply placed a stick of deodorant near the inlet to the mass spectrometer.[65] Another lock mass approach was proposed by Wenger and Coon that employed a proportional m/z correction using fluoranthene cations generated from the chemical ionization reaction in the ETD chamber as an internal calibrant.[66] This approach highlights several advantages in that the calibration method is not instrument specific, given that it is a proportional correction in m/z space, and the internal calibrants are created within the instrument so the common drawbacks of using internal calibrants (e.g. charge competition and ion suppression between the analyte and calibrant) are avoided. Recently, Gorshkov has demonstrated that space charge effects can be accounted for by introducing a two parameter calibration including a term that is proportional to the square root of the AGC target for the Orbitrap.[64]

Matrix-assisted laser desorption electrospray ionization (MALDESI) was introduced in 2006 as a hybrid ambient ionization source that uses a laser to resonantly excite an endogenous or exogenous matrix to facilitate the desorption of neutral material that is post-ionized by electrospray ionization (ESI).[67] The description of this process is independent of laser wavelength given that one would choose a matrix which strongly absorbs in the wavelength region of the laser emission. The imaging capabilities of the IR-MALDESI source have recently been summarized including the use of exogenous ice as a matrix and synchronization between the pulsing of the laser and collection of mass spectra to greatly improve spot-to-spot reproducibility.[68] Herein we describe the application of a cyclotron frequency correction to account for space charge induced frequency shifts that are observed during an IR-MALDESI MSI experiment coupled to an FT-ICR mass spectrometer. The frequency correction is determined by calculating the average offset of the observed frequency from the expected frequency for a series of PDMS ions. This correction provided part per billion mass accuracy and was the most precise when compared with a few other calibration techniques.

EXPERIMENTAL

Materials

Formic acid was purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile and water were purchased from Burdick & Jackson (Muskegon, MI, USA). All materials were used as received without further purification.

Methods

The electrospray solution was prepared by mixing one part acetonitrile and one part water (v/v) with 0.1 % formic acid. Liver tissue was obtained from a laboratory animal dosed with the pharmaceutical Tykerb. Mouse brain tissue was snap frozen in liquid nitrogen and stored

at -80°C . Both tissue types were sectioned on a cryostat into $50\text{ }\mu\text{m}$ sections that were thaw-mounted onto a glass microscope slide.

LTQ-FT Ultra Mass Spectrometer

The IR-MALDESI source was coupled to a Thermo Scientific LTQ-FT Ultra mass spectrometer (Thermo Scientific, West Palm Beach, FL, USA) that is fitted with an actively shielded 7 T superconducting magnet. The AGC was not applicable to the imaging experiments due to the pulsed nature of the source and was turned off. The ion injection time (IT) determines the length of time the ions are accumulated in the ion trap and this value was set to 200 ms in order to collect the ions generated from the three laser pulses at 20 Hz fired at each spot (pixel), which would take roughly 150 ms. Full mass spectra (m/z 190-1900) were acquired at 100000 RP (FWHM) at each pixel, though for the IR-MALDESI imaging experiments, tissue related ions are typically observed between m/z 200-1200.

IR-MALDESI Imaging

A more detailed description of the optimized IR-MALDESI source parameters as well as the imaging source parameters has been provided previously.[68-69] In short a thin layer of ice is deposited on the tissue section using a liquid cooled Peltier stage. A wavelength tunable mid-IR laser (IR-Opolette, Opotek, Carlsbad, CA) is pulsed into the sample to excite the ice matrix and facilitate the desorption of tissue related material. This desorbed material, consisting primarily of neutral molecules and particulate matter, partitions into an orthogonal plume of charged electrospray droplets and is post-ionized through an ESI-like process. The IR-MALDESI imaging source allows for synchronization of the triggering of both the laser and mass spectrometer.[68]

Data Analysis

The .RAW files were converted to mzXML using the freely available conversion tool (<http://proteowizard.sourceforge.net/index.shtml>).[70] A MATLAB® (The MathWorks Inc, Natick, MA) script was written to import the mzXML file and obtain the centroid mass and intensity for a list of ions from each individual spectrum. These ions included several protonated or ammonium adducted ambient ions that are used as internal calibrants, the protonated ion for Tykerb, protonated cholesterol after water loss, and a protonated lipid (PC(38:4) or PE(41:4)) at the higher end of the mass range. Table 1 lists these ions, their chemical formulas, and exact masses. The tissue related peaks (Tykerb, cholesterol, and the protonated lipid) are homogeneously distributed throughout the liver tissue section as determined from their ion maps from the imaging experiment (data not shown). The centroid algorithm used to calculate the observed m/z is based on fitting a parabola to three points of each peak (local maxima and adjacent datapoints) has been described previously.[71-72] The calculated centroid for each peak was identical to those calculated by the instrument software (Thermo Xcalibur). The back conversion from m/z space to frequency space was performed using the instrument's external calibration parameters which can be found in the header of the raw file. Even with the AGC off, the instrument will use the calibration parameters that were determined for the AGC target that is set. After extraction of this information, several different calibration methods were performed and their effect on mass accuracy was evaluated by plotting cumulative frequency and a scatter plot with color-coded density that was designed in MATLAB®.

For the generation of the ion maps the mzXML files were loaded into the MSiReader software (available at www.msireader.com), a MATLAB® based software program which was developed in-house. This software is capable of generating ion maps from high resolution MS data. The images that are displayed in this manuscript have been linearly interpolated to an order of 2.[73]

RESULTS and DISCUSSION

Frequency shift recalibration

With no direct control over the magnitude of the ion population from spectrum to spectrum, space charge effects will result in systematic fluctuations in the observed cyclotron frequency. A number of groups have shown that this frequency shift is linearly dependent on the ion population and that the primary contribution to the shift is independent of m/z . [12-15] Despite this observation, a large portion of the proposed recalibration techniques involve adding new parameters or calculating new calibration constants to account for the shift in frequency.[29-36, 42-43] If the magnitude of this shift can be quantified, then correcting for m/z -independent space charge effects could be as straightforward as adding that shift to all of the observed frequencies then converting into m/z space. However, the quantification of the space charge induced frequency shift would require some form of internal standard. With ESI and ESI-based ambient ionization techniques, such as IR-MALDESI, PDMS molecules present in the ambient laboratory readily ionize and are consistently present in each mass spectrum. Despite initially being viewed as a contaminant, [62] the use of these ions as internal calibrants is becoming more prevalent.[63] In our laboratory a distribution of several of these ambient ions are commonly observed in every IR-MALDESI spectrum. Table 1 shows those ambient ions that are typically most abundant, including the protonated and ammonium adducted heptamer and octamer. A key feature of IR-MALDESI is the option to add internal calibrants to the electrospray solution without directly interfering with the sample.[74] However, this was not necessary given that the four PDMS ions were consistently observed in all 5,000 mass spectra from the tissue imaging experiment.

A schematic of the proposed calibration technique using these ions as internal calibrants is shown in Figure 1. For each scan, the observed m/z values are converted back into frequency space using the instruments external calibration equation. The exact mass of each of the ambient ions listed in Table 1 are also converted into frequency space using this external calibration equation. The difference between the exact and observed frequencies for each of the ambient ions is then determined (Δf). The average of these four values represents the average space charge induced frequency shift (avg Δf) that is then added to all of the observed frequencies. These calibrated frequencies are converted back into m/z space using the instruments external calibration equation to obtain a calibrated mass spectrum. This workflow is then repeated for every mass spectrum. Using a MATLAB® script for the average frequency shift recalibration routine, an imaging experiment with 5000 scans can be processed in a little over two minutes. An example calculation of this workflow for one mass spectrum can be found in the supplementary material.

In order to evaluate the effectiveness of this calibration, the MMA of the PDMS ions as well as several tissue related ions was determined for the externally calibrated spectra and the spectra calibrated using the observed frequency shift. It is important to note that only the ambient ions in Table 1 were used as internal calibrants and the tissue related ions were used to identify the effectiveness of the calibration. Figure 2 shows scatter plots of the MMA for these ions with respect to the total ion current (TIC) along with cumulative percentage plots for both calibrations. Given the large number of data points in the scatter plots (roughly 38,000 points), a majority of them overlap. To account for this, the data was distributed into 500 equally spaced bins along each axis (histograms are shown adjacent to each axis). The points were then color coded according to the number of points in each bin of the two dimensional histogram to illustrate the density of the data points. Even with the AGC off, the external calibration (Figure 2a) still maintained a majority of the data within 2 ppm. The average MMA for the externally calibrated spectra was 750 ppb with a standard deviation of 430 ppb. However, there is a systematic positive shift in MMA with increasing ion current

which most likely caused by space charge effects. This shift is corrected for using the proposed calibration as seen in the centering of a majority of the points around zero even at high TIC. The average MMA for this method was found to be 38 ppb with a standard deviation of 250 ppb.

Comparison with other calibration methods

As alluded to earlier, there have been a large number of calibration techniques proposed in the literature. In order to evaluate the average ambient ion frequency shift calibration, the same data set was calibrated using several of these other methods. The results of this comparison are shown in Table 2. The scatter plots, similar to those shown in Figure 2, for each of these calibration methods can be found in the supplementary material. Each of the calibration techniques tested were able to achieve an average MMA in the ppb range with varying degrees of precision. The most accurate calibration method was the altered Francl calibration (method 6 in Table 2), proposed by Muddiman and coworkers, that includes an additional term to account for the individual ion abundance (I).[33] While this method was the most accurate, it was also the least precise with a standard deviation that was almost twice as large as the external calibration. The modified Francl equation, also proposed by Muddiman, that includes terms to account for individual ion abundance as well as total ion abundance (method 5 in Table 2) has recently been used for recalibrating a FT-ICR imaging dataset.[75] Smith et al. found this method to provide the highest mass accuracy; however the technique proposed here (method 3 in Table 2) is shown to provide significant improvement in both mass measurement accuracy and precision when compared to each of the methods that were tested. One possible explanation for the improvement that is realized using the frequency shift recalibration is that it is a direct correction in frequency space, whereas the other techniques utilize intensity measurements to adjust the m/z scale. The measurement of intensity is not necessarily accurate and thus the error in the intensity measurement can propagate into the error of the mass measurement when utilizing total ion current or individual ion abundance for mass calibration. Another interesting observation is demonstrated in the comparison of methods 2 and 3 where the determination of the frequency shift using a series of ambient ions is significantly more accurate and precise than relying on a single ion. It should also be noted that while ambient PDMS ions are used here as internal calibrants, this calibration method should work for any series of internal calibrants. In addition, this method of determining the frequency shift using internal calibrants could also be applied to calibration of the Orbitrap. However, since the Orbitrap is externally calibrated using a single parameter equation, this would likely give results that are similar to the lock mass function that is already integrated into the Orbitrap software.

High resolving power mass spectrometry imaging

In mass spectrometry imaging of biological tissue sections, a large number of ions spanning a variety of molecular classes (metabolites, lipids, glycans, proteins, etc.) can be detected simultaneously. Given that chromatographic separation of these species is not typically feasible; the RP of the mass spectrometer is relied upon to resolve these species through separation by m/z . An example of the importance of using high RP and accurate mass measurements for mass spectrometry imaging is demonstrated in Figure 3. The black trace is a portion of a mass spectrum from an IR-MALDESI FT-ICR MSI analysis of a mouse brain tissue section. The two peaks correspond to two phospholipid species that are resolved at 100,000 RP (FWHM at m/z 400). These two lipids have dramatically different spatial distributions in the section that was analyzed (black boxes in Figure 3). The accurate mass obtained from the centroid of these peaks leads to their preliminary identification as PE(27:3)/PC(24:3) for 756.5551 and PE(38:2)/PC(35:2) for 756.5921. The red trace represents a theoretical spectrum of these two peaks at 15,000 RP (FWHM at m/z 400). At the lower RP, these peaks are unresolved and combined under the same peak. The spatial

distribution would then appear as a sum of both peaks (red box in Figure 3) which demonstrates the loss of spatial information from the latter peak. More importantly, the centroid of this peak would then correlate more closely to a species that is not actually present. In addition, the average frequency shift recalibration was also performed on this mouse brain data set (data not shown). For both peaks shown in Figure 3 the average mass accuracy was improved from roughly 1.3 ppm with the external calibration to about 0.7 ppm with the proposed calibration, thereby improving the confidence of the identification. This also shows that the calibration routine works equally well for species that may not be homogeneously distributed.

CONCLUSIONS

It has been shown that an increase in the total ion population can result in a systematic shift in the observed cyclotron frequency in FT-ICR. Without direct control over the size of this ion population from scan to scan, external calibration can lead to significant errors in mass measurement. The calibration technique that is described here uses a series of ambient PDMS ions as internal calibrants to quantify this space charge induced frequency shift allowing for proper adjustment of the observed cyclotron frequency. This method is capable of achieving part per billion mass accuracy with the highest precision of the calibration techniques tested for an IR-MALDESI imaging dataset. Also, given that this calibration routine is executed in MATLAB®, we are working on integrating this workflow and perhaps other universal mass calibration techniques[66] into our freely available and vendor neutral MSI analysis software, MSiReader.[73]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Typical workflow of the average frequency shift recalibration technique that involve conversion of each mass spectrum into frequency space, determination of the average frequency shift, correction of the shift, and conversion back to m/z space.

Figure 2.

Scatter plots of the mass accuracy against total ion current (TIC) of the PDMS ions as well as several tissue related ions over the course of an IR-MALDESI imaging experiment. a) The external calibration with the AGC off shows a systematic deterioration in mass accuracy with increasing total ion current indicating a frequency shift due to space charge effects. b) Correcting for this frequency shift using the average ambient ion frequency shift calibration, the points are more closely centered around zero and the mass shift associated with the space charge effects is reduced.

Figure 3.
Demonstration of the importance of high resolving power and accurate mass measurements in mass spectrometry imaging.

Table 1

The formula, observed ion, and exact ion mass for the several PDMS ions that were used as internal calibrants and three homogenously distributed tissue related species.

Ion Type	Ion	Formula	Adduct	Ion Exact Mass
Ambient Ions (Internal Calibrants)	Heptadimethylcyclsiloxane	C ₁₄ H ₄₂ O ₇ Si ₇	[M+H] ⁺	519.1388 ₂
	Heptadimethylcyclsiloxane	C ₁₄ H ₄₂ O ₇ Si ₇	[M+NH ₄] ⁺	536.1653 ₇
	Octadimethylcyclsiloxane	C ₁₆ H ₄₈ O ₈ Si ₈	[M+H] ⁺	593.1576 ₁
	Octadimethylcyclsiloxane	C ₁₆ H ₄₈ O ₈ Si ₈	[M+NH ₄] ⁺	610.1841 ₆
Tissue Related Ions	Cholesterol	C ₂₇ H ₄₆ O	[M+H-H ₂ O] ⁺	369.3515 ₈
	Tykerb	C ₂₉ H ₂₆ ClFN ₄ O ₄ S	[M+H] ⁺	581.1420 ₁
	PE(41:4) / PC(38:4)	C ₄₆ H ₈₄ NO ₈ P	[M+H] ⁺	810.6007 ₃

Table 2

Comparison of several calibration methods.

Method	Calibration Equation	Avg MMA (ppm)	SD (ppm)	Reference
1	$\frac{m}{z} = \frac{A}{f} + \frac{B}{f^2}$	0.750	0.430	26
2	$\frac{m}{z} = \frac{A}{f_0 + \Delta f} + \frac{B}{(f_0 + \Delta f)^2}$	0.175	0.316	This work
3	$\frac{m}{z} = \frac{A}{f_0 + \text{avg}\Delta f} + \frac{B}{(f_0 + \text{avg}\Delta f)^2}$	0.038	0.250	This work
4	$\frac{m}{z} = \frac{A}{f} + \frac{B}{f^2} + \frac{C \times \text{TIC}}{f^2}$	0.085	0.326	42
5	$\frac{m}{z} = \frac{B}{f - (A + C \times I + D \times \text{TIC})}$	0.052	0.471	33 *
6	$\frac{m}{z} = \frac{B}{f - (A + C \times I)}$	-0.006	0.844	33 *
7	$\frac{m}{z} = \left(\frac{m}{z}\right)_{\text{observed}} \times \left[\frac{\left(\frac{m}{z}\right)_{\text{exact}}}{\left(\frac{m}{z}\right)_{\text{observed}} \big _{\text{calibrant}}} \right]$	0.208	0.360	67 *

* The terms in these equations have been modified to correlate with the terminology of the manuscript.