Quantitative MALDI Tandem Mass Spectrometric Imaging of Cocaine from Brain Tissue with a Deuterated Internal Standard

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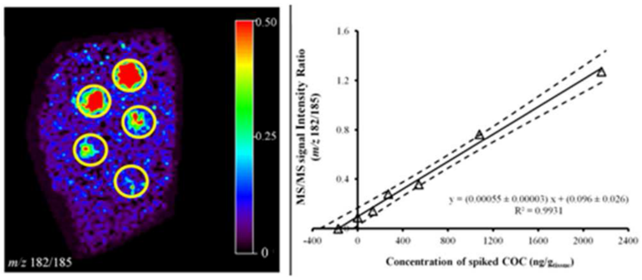
ABSTRACT: Mass spectrometric imaging (MSI) is an analytical technique used to determine the distribution of individual analytes within a given sample. A wide array of analytes and samples can be investigated by MSI, including drug distribution in rats, lipid analysis from brain tissue, protein differentiation in tumors, and plant metabolite distributions. Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique capable of desorbing and ionizing a large range of compounds, and it is the most common ionization source used in MSI. MALDI mass spectrometry (MS) is generally considered to be a qualitative analytical technique because of significant ion-signal variability. Consequently, MSI is also thought to be a qualitative technique because of the quantitative limitations of MALDI coupled with the homogeneity of tissue sections inherent in an MSI experiment. Thus, conclusions based on MS images are often limited by the inability to correlate ion signal increases with actual concentration increases. Here, we report a quantitative MSI method for the analysis of cocaine (COC) from brain tissue using a deuterated internal standard (COC-d_3) combined with wide-isolation MS/MS for analysis of the tissue extracts with scan-by-scan COC-to-COC-d_3 normalization. This resulted in significant improvements in signal reproducibility and calibration curve linearity. Quantitative results from the MSI experiments were compared with quantitative results from liquid chromatography (LC)-MS/MS results from brain tissue extracts. Two different quantitative MSI techniques (standard addition and external calibration) produced quantitative results comparable to LC-MS/MS data. Tissue extracts were also analyzed by MALDI wide-isolation MS/MS, and quantitative results were nearly identical to those from LC-MS/MS. These results clearly demonstrate the necessity for an internal standard for quantitative MSI experiments.



Mass spectrometric imaging (MSI) has rapidly expanded from early applications in imaging of proteins[1,2] into numerous compound classes including lipids,[3] pharmaceuticals,[4] and endogenous metabolites.[5,6] The technique generates two-dimensional (or, more recently, three-dimensional) images of the distribution and concentration of analytes in a tissue section.[7,8] The quantitative limitations of matrix-assisted laser desorption/ionization (MALDI) MSI have recently come under scrutiny,[9] as MSI techniques suffer from two major limitations: the inability to control for tissue-specific ion suppression and the irreproducibility of ion signals from scan to scan.[10] These limitations can be caused by numerous factors, including tissue heterogeneity, matrix crystal heterogeneity, laser power fluctuations, and tissue-specific ion suppression, and preclude in situ quantification.[11,12] To date, quantitative MSI applications have generally been limited to relative determinations that require verification from other analytical techniques, such as liquid chromatography—tandem mass spectrometry (LC-MS/MS).[13]

Quantitative MS analytical techniques typically rely on internal standards to control for sources of error arising from sample preparation, injection-volume irreproducibility, or ion-source variability. In most instances, an internal standard is added in a constant amount to the samples being analyzed prior to any additional sample preparation. Applying an internal standard to control for the previously listed limitations could improve the quantitative capabilities of MSI. Utilizing an internal standard for traditional MALDI analysis has been shown to improve quantitation;[14−17] however, these methods simply add the internal standard to the sample droplet, which is not practical in a tissue imaging experiment. For MSI, the internal standard must be applied uniformly to the tissue by either microspotting or spray-coating. In this work, the internal standard was applied with a modified ink-jet printer[18] that has been shown to produce a uniform layer.[19] To best mimic the extraction of an analyte from the tissue, the internal standard was applied beneath the tissue (to the glass slide before placement of the tissue) and then was extracted through the tissue and into the matrix crystals. For the quantitative analysis of cocaine (COC) in nucleus accumbens tissue, deuterated cocaine (COC-d_3) was used as an internal standard. Deuterated analogues routinely perform best as an internal standard, as they have chemical characteristics nearly identical to those of their unlabeled counterparts. Applying an internal standard in this manner has been shown to improve scan-to-scan reproducibility and calibration-curve linearity for quantitative imaging applications.[19] COC detection from nucleus accumbens tissue was chosen as a model system, as comparable extraction techniques for LC-MS have been well documented and, therefore, can be used to supplement quantitative MSI experiments.[12,20−22] Tissue levels of recreationally abused drugs such as cocaine are more representative of the dose upon time of death than are levels in blood or urine. Furthermore, mass spectrometric imaging methods could allow toxicology laboratories to circumvent extraction and chromatography methodologies.[12]

Recently, an MSI experiment reported the quantitative distributions of tiotropium bromide and imipramine in lung tissue.[10,23] The quantitative estimations were made on the basis of calibration standards pipetted atop a control tissue slice, by comparing the signal of the calibration standard to the positive sample. Spotting standards atop a tissue section might not accurately reflect the drug's signal from within the tissue, as the analyte's extraction efficiency might vary. As we and others have observed, the diameter and uniformity of the standard spot, and thus the concentration of standards spotted atop the tissue, are also difficult to control.[10,19] These experiments, however, do show progress in the area of quantitative MSI, as the authors were able to produce a linear calibration curve from the spotted samples, from which we were then able to make a quantitative estimation of the administered drug. Another recently published study by Hankin and Murphy[24] examined the relationship between MALDI MSI signals and lipid concentrations in brain tissue and succeeded in making relative quantitative comparisons between MSI and LC-MS/MS results. However, these comparisons were made with extensive signal normalization and baseline correction; indeed, the authors concluded that MSI results should be taken with caution because of the complex environment of direct tissue analysis.[24]

Normalizing the ion signal of the target analyte to that of an internal standard has been suggested to further improve MSI quantitation and help identify tissue-specific ion suppression.[25] Scan-by-scan normalization by a deuterated internal standard for MALDI MSI has recently been shown to improve ion-signal reproducibility, improve image quality,[19] and significantly improve the quantitative ability of MALDI MSI.[10,12,26] Because the internal standard must have similar extraction, cocrystallization, and ionization properties as the target analyte for quantitation, a deuterated analogue is an ideal option. The internal standard can be applied to the tissue in three ways: (1) by applying a mixture of the internal standard and MALDI matrix to the tissue,[27] (2) by applying an internal standard atop the tissue prior to applying the matrix,[25] or (3) by applying the internal standard beneath the tissue sample prior to placing the tissue and applying the matrix.[18] Applying the internal standard along with the matrix has been shown to account for tissue-specific ion suppression;[27] however, we have shown that applying the internal standard mixed into the matrix solution neither improves image quality nor reduces signal variation.[28] Issues for quantitation can arise if the matrix/internal standard solution is applied manually, as there might be uneven matrix/internal standard application, resulting in unaccountable analyte-to-internal-standard ratio differences. Applying an internal standard atop the tissue might not accurately reflect the analyte being extracted from within or beneath the tissue sample, as the internal standard might reside atop the tissue. Applying the internal standard beneath the tissue might best reflect the detected ion signal of the target analyte within the tissue as well as those of calibration standards that have also been applied beneath the tissue, as the dried standards are "extracted" into the wet tissue when the tissue section is placed atop the dried standards. Both the target analyte and the internal standard are then further extracted and cocrystallized from the tissue sample during matrix application. This approach has been shown to help control for tissue variation as well as extraction differences from different tissue types.[27] Finally, because of the significant amount of signal variation in MALDI, the internal standard and analyte should be detected in the same analytical MS^n scan. Detecting the analyte and internal standard in separate scans has been shown to offer no quantitative advantage because of the signal variation from the MALDI process.[13] For MS applications, this is easily done by detecting a mass range large enough to incorporate both ions; however, for tandem mass spectrometry (MS/MS) applications, a wide-isolation window that includes both the analyte and the internal standard must be utilized.[12]

By combining the advantages of a uniformly applied internal standard beneath a tissue sample with the specificity of wide-isolation MS/MS[11] and scan-by-scan COC-to-COC-d_3 normalization, absolute quantitative MSI was performed for the analysis of COC in a brain tissue section. These results were compared to LC-MS/MS and MALDI MS/MS results from tissue extracts of the same brain sample. This study expands and improves on previously published methods[26,28] by evaluating both ion-trap and quadrupole-time-of-flight (Q-TOF) instrumental approaches as well as by utilizing quantitation through both standard additions and external calibration. Finally, the methods developed for quantitative imaging were used successfully to quantify COC extracted from brain tissue in parallel to LC-MS/MS experiments.

■ MATERIALS AND METHODS

Chemicals. Standards of cocaine (COC, MW 303) and deuterated cocaine (COC-d_3, MW 307) were purchased from Ceriliant (Round Rock, TX, USA). Superfrost glass plus microscope slides and LC-grade methanol (MeOH), acetonitrile (ACN), and water (H_2O) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Stainless steel microscope slides were purchased from Thermo Scientific (San Jose, CA, USA). For MSI analysis, working standards of COC and COC-d_3 were diluted to 10 ng/μL in ACN for LC-MS/MS analysis and to 10 ng/μL in 50/50 MeOH/H_2O (v/v) for MSI. Calibration standards of COC used for MALDI imaging were made to 0.030, 0.060, 0.13, 0.25, and 0.50 ng/μL in 50/50 ACN/H_2O (v/v), and 1 μL of each calibration standard was pipetted onto a stainless steel microscope slide prior to COC-d_3 application. Tissue concentrations were estimated from the area of the detected signal and the thickness of the tissue, assuming that the density of wet tissue is ~1.05 g/mL.[18] The MALDI matrix 2,5-dihydroxybenzoic acid (DHB) was purchased from Acros Organics (Geel, Belgium) and prepared at a concentration of 20 mg/mL in 70/30 MeOH/H_2O (v/v).

For LC-MS/MS, working standards of COC were prepared to 0.50 and 5.0 ng/μL, and working standards of COC-d_3 were prepared to 0.50 ng/μL. Calibration standards were spiked into control brain tissue during tissue homogenization at concentrations of 50.0, 100, 200, 400, 1,000, and 2,000 $ng_{COC}/g_{wet\ tissue}$, and the internal standard was spiked at 40 $ng_{COC\text{-}d_3}/g_{wet\ tissue}$.

Tissue Preparation for MSI. For quantitative MSI, calibration standards of COC in $MeOH/H_2O$ were pipetted onto a microscope slide and allowed to dry under ambient conditions. An Epson (Long Beach, CA, USA) Stylus R280 ink-jet printer was used to apply three coats of the COC-d_3 in $MeOH/H_2O$ to the microscope slides. Positive and negative control (with and without COC, respectively) samples of human nucleus accumbens (NA) excised from the brain were provided by the El Paso County Coroner's Office in Colorado Springs, CO, USA. The NA was extracted during autopsy, snap frozen in liquid nitrogen, and stored at −80 °C. Sectioning of the brain tissue was performed on a Microm HM 505E cryostat (Waldorf, Germany). Tissue was sliced at −25 °C to a thickness of 20 μm and thaw-mounted onto the microscope slides previously coated with COC calibration standards and COC-d_3. DHB was then applied to the tissue samples using the ink-jet printer.[17] As in any experiments that employ human specimens, appropriate blood-borne pathogen safety precautions should be followed. Also, application of matrix solutions should be performed in a hood.

MSI Quantitation. For the MSI experiments, all mass spectrometric images were acquired with either a Synapt MALDI-Q-ion mobility (T-wave cell) time-of-flight (Q-TOF) instrument equipped with a 200-Hz, 355-nm Nd:YAG laser at a frequency of 200 Hz and spot diameter of 150 μm (Waters Corporation; Milford, MA, USA) or a MALDI LTQ XL linear ion trap equipped with a 60-Hz, 337-nm nitrogen laser with a spot diameter of 100 μm (Thermo Corp. San Jose, CA, USA). The Q-TOF instrument was operated in MS/MS mode with the isolation width of the quadrupole increased to 6 Da to transmit the $[M + H]^+$ ions of both COC (*m/z* 304) and COC-d_3 (*m/z* 307) prior to fragmentation. Sample slides were inserted into the vacuum chamber and moved with respect to a fixed laser at a step size of 250 μm, with a laser spot size of 150 μm. MS/MS spectra were summed from 200 laser shots at each raster step. For MS/MS imaging experiments on the LTQ instrument, the isolation window was centered at *m/z* 305.5 ± 3 to trap the $[M + H]^+$ ions of both COC and COC-d_3 prior to fragmentation. Upon insertion of the samples into the vacuum chamber, the sample was moved with respect to a fixed laser at a step size of 250 μm, with a laser spot size of 100 μm, and MS/MS spectra were collected using eight laser shots at each raster step. The ions of interest were fragmented in both instruments by collision-induced dissociation (CID), and the fragments of each precursor ion were monitored: for COC, *m/z* 304 → 182; for COC-d_3, *m/z* 307 → 185. Resulting MS/MS data from the Q-TOF instrument were processed using BioMap (Novartis, Basel, Switzerland), and images from the LTQ instrument were processed with Qual Browser (Thermo Scientific) and Surfer 8 (Golden Software, Golden, CO, USA). Normalized images were generated using the divide function in BioMap to generate COC/COC-d_3 (*m/z* 182/185) images. Regions of interest (ROI) were drawn using the BioMap program, and from these regions, the average *m/z* 182/185 ratios and standard deviations were calculated and plotted on a standard additions curve. By plotting the *m/z* 182/185 ratio as a function of position, normalized images from the LTQ instrument were generated in Surfer 8 from data extracted from Qual Browser. For quantitative determinations of COC, a standard additions analysis was performed using the Q-TOF instrument and the calculated product-ion ratio of COC/COC-d_3 from the spotted COC calibration standards beneath the positive tissue. Quantitation by external calibration was also performed with calibration standards spotted beneath a control tissue section and a quality-control (QC) standard of COC (0.25 ng/μL) spotted beneath a positive tissue section to ensure that the ion signals were comparable between tissues. The resulting signal from the COC was compared to the calibration curve calculated from the control tissue. All quantitative MSI data were then compared to LC-MS/MS and MALDI-MS/MS data from brain tissue extracts.

Tissue Extraction/Quantitation. Nucleus accumbens samples were extracted using manual tissue homogenization and solid-phase extraction (SPE) according to the procedure published by Lowe et al.[20] Two 100-mg sections of the positive COC tissue samples were dissected and extracted. The extracts were compared to calibration sample extracts from 100-mg sections of the negative control sample. The total sample makeup was two positive samples, six calibration samples, and one negative control sample. Calibration samples were prepared at the concentrations previously stated, and following SPE, the samples were reconstituted in 100 μL of H_2O/ACN 90/10 (v/v). A 5-μL aliquot was taken for wide-isolation MALDI-MS/MS analysis. The remaining 95 μL of each sample was submitted for triplicate LC-MS/MS analysis on a Thermo Scientific TSQ 7000 triple-quadrupole mass spectrometer (QqQ) equipped with an Agilent LC system (Santa Clara, CA, USA). Mobile phase A was composed of H_2O with 0.1% formic acid; mobile phase B was composed of ACN with 0.1% formic acid. A gradient LC program was utilized starting with 5 min of 10% B, then a ramp to 90% over 8 min with a 3 min hold at 90%, and re-equilibration for 3 min at 10% B. A 5-μL aliquot of each extract were loaded onto the LC column followed by MS/MS analysis by positive-mode multiple-reaction monitoring (MRM) for COC (*m/z* 304 → 182) and COC-d_3 (*m/z* 307 → 185) for quantitative analysis.

MALDI-MS/MS of the 5-μL aliquots was performed with a Thermo Scientific MALDI LTQ XL instrument utilizing a 6-Da wide-isolation window centered at *m/z* 305.5 with scan-by-scan COC-to-COC-d_3 normalization. From the 5-μL aliquot, 1 μL of each sample (two positive samples, six calibration samples, and one negative control) was spotted in duplicate onto a glass plus microscope slide. Then, 40 mg/mL of DHB dissolved in $MeOH/H_2O$ 70/30 (v/v) was applied uniformly to the slide using a Meinhard nebulizer (Golden, CO, USA) at a flow rate of 2.4 mL/min. Within each spotted sample, two areas of 50 MS scans were analyzed by MALDI-MS/MS with a step size of 200 μm, a laser energy of 14 μJ, and eight laser shots per step. This resulted in quadruplicate analysis of each sample. From each 50-scan MS/MS experiment, the extracted ion currents for COC (*m/z* 304 → 182) and COC-d_3 (*m/z* 307 → 185) were plotted using data extracted to Microsoft Excel from Qual Browser. From each MS/MS experiment, the *m/z* 182/185 ratio was calculated, averaged, and used for quantitation.

■ RESULTS AND DISCUSSION

To demonstrate successful quantitation of COC from brain tissue by MSI, results from imaging experiments can be validated with quantitative results using accepted quantitative analytical techniques such as LC-MS/MS. Four requirements have been suggested[22] for successful quantitation of small molecules by MALDI: a high-repetition-rate laser (for analysis

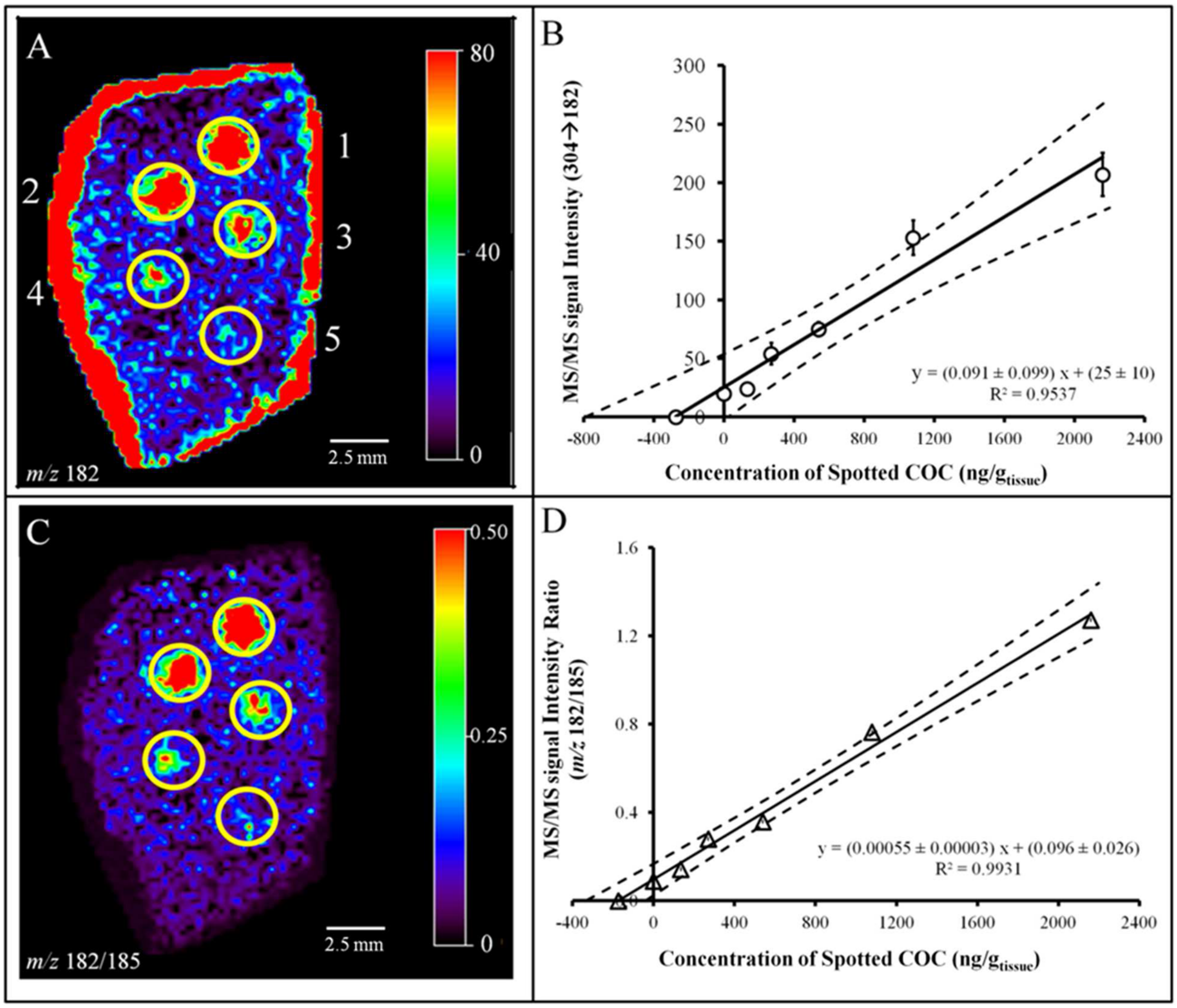


Figure 1. Wide-isolation MS/MS imaging experiment on the Q-TOF instrument for the quantitative analysis of COC from nucleus accumbens. (A) Ion intensity of *m/z* 182 and (C) the ratio of *m/z* 182/185 were used to generate MS/MS images. Calibration spots increase in concentration from 200 (spot 5) to 2200 ng/g_{tissue} (spot 1). Corresponding standard addition plots for quantitation with (B) *m/z* 182 ion intensity and (D) the ratio of *m/z* 182/185. Normalization to the COC-d_3 improved the linearity (as indicated by the R^2 value) and the error of the calibration plot (as indicated by the relative standard deviation of the slope and intercept), leading to improved quantitative estimation of COC in the tissue. Confidence bands were calculated from the 95% confidence interval, and error bars represent the standard deviations of the mean.

with a QqQ system), tandem MS (for increased selectivity), sample cleanup (for proper cocrystallization), and an internal standard (for increased precision). In these MSI experiments, these requirements were addressed by employing wide-isolation MS/MS imaging experiments that allow for scan-by-scan MS/MS ion-signal normalization of the COC ion signal by the internal standard ion signal, COC-d_3. With wide-isolation MS/MS, the advantages of MS/MS are preserved (improved selectivity and sensitivity), and the quantitation and ion-signal reproducibility of MALDI are also improved through the scan-by-scan normalization of the analyte to the internal standard.[12] Scan-by-scan ion-signal normalization circumvents the need for a high-repetition-rate laser and has been shown to improve signal reproducibility 5-fold. Cocrystallization of the target analyte (COC) and internal standard (COC-d_3) was enhanced by applying the MALDI matrix in a uniform manner with an ink-jet printer.[18] In MSI, cocrystallization of the target analytes can also be enhanced by improved extraction of the analyte from the tissue with the matrix solvent. Improving the extraction and cocrystallization of the target analytes can lead to improved sensitivity. In this study, we show extraction of spiked COC and COC-d_3 from beneath the tissue; however, this extraction could be further optimized by evaluating other matrix solvents and the effect of tissue thickness.

Quantitative Detection of COC by MS/MS Imaging. Adapting a wide-isolation MS/MS approach developed for the LTQ instrument allowed for simultaneous MS/MS detection of COC and COC-d_3 with the Q-TOF instrument. This was accomplished by decreasing the resolution of the resolving quadrupole to include both COC (*m/z* 304) and COC-d_3 (*m/z* 307); after CID, product ions from both were detected in a single TOF spectrum (COC, *m/z* 304 → 182; COC-d_3, *m/z* 307 → 185), in agreement with previously published results.[12]

This wide-isolation MS/MS approach with the Q-TOF instrument was used to generate images from either the intensity of *m/z* 182 (Figure 1A) or the ratio of *m/z* 182/185 intensities determined from the MS/MS spectrum (Figure 1C). The calibration standards of COC spotted beneath the sample were used to quantify the COC previously present in the tissue by extrapolation of the standard additions plot in Figure 1B to the *x* axis. The MS/MS images of the product ion intensity of *m*/*z* 182 (Figure 1A), the normalized COC/COC-d_3 (*m*/*z* 182/185) image (Figure 1C), and the corresponding standard addition plots (Figure 1B,D, respectively) were generated from the wide-isolation MS/MS experiment performed on the positive COC section of nucleus accumbens. Normalizing the COC/COC-d_3 ion-signal ratio for quantitative analysis improved the linear correlation coefficient (R^2) while decreasing the width of the 95% confidence limits by a factor of 2 and decreasing the relative uncertainty in the best-fit slope by a factor of 20. These improvements are a reflection of the improved ion-signal reproducibility observed upon monitoring the ion-signal ratio of analyte to internal standard.

Analysis of three serial tissue sections with the Q-TOF instrument by standard addition exemplified in Figure 1D yielded an average COC concentration of 240 ± 100 ng/g_{tissue}. The improvements in calibration-curve linearity and signal reproducibility from normalizing by the internal standard can be attributed to an improvement in scan-to-scan reproducibility as well as control for extraction and ionization variability resulting from differences in tissue makeup across the sample. Scan-to-scan signal variation and back-extrapolation to the *x* axis contribute significantly to the calculated error. Also, error in the calculated concentration of the spotted COC standards can affect the resulting quantitative COC determination. The calculated COC tissue concentration of standards relies on two assumptions: (1) the spotted COC is distributed uniformly across the calibration spots and (2) the diameter of the applied COC spot remains consistent for the calibration spots (minimal lateral diffusion). How evenly the spotted COC standard is distributed through the tissue has proven difficult to determine; however, as shown in Figure 1A,C, signal was detected in areas of the spotted COC standards indicating extraction. To estimate extraction efficiency through the tissue, one could compare the COC-d_3 signal detected in the tissue to the detected COC-d_3 signal in areas outside the tissue slice; the COC-d_3 signal on the tissue was typically 1−2% of that off the tissue. However, this reduction in ion signal may not reflect just extraction efficiency but also differences in ionization efficiency between COC-d_3 on the glass slide and on the tissue. Standards of COC could also be spotted atop the tissue; however, this may not accurately reflect extraction efficiency of standards spotted beneath tissue, because the COC spotted atop tissue may simply remain atop the tissue and not distribute evenly through the thickness of the tissue sample. Scan-by-scan normalization by a deuterated internal standard that has also been extracted through the tissue (from beneath the tissues) is the best method of signal normalization and quantitation, as the internal standard behaves similarly to the target analyte in terms of extraction, cocrystallization, and ionization efficiency.

Concentration of COC was also measured with the Q-TOF instrument by comparing the COC/COC-d_3 signal ratio from the positive tissue to the spotted COC standards beneath a control section of tissue (external calibration). The resulting calibration plot for the MS/MS *m*/*z* 182/185 intensity ratio is shown in Figure 2. The spotted concentration of the applied quality-control (QC) sample was ~4000 ng_{COC}/g_{tissue}, determined from the area of the applied COC standard assuming even extraction into the 20-μm-thick tissue. Calculating the concentration of the QC from the positive sample from the calibration curve generated from the control tissue by using the *m*/*z* 182/185 ion ratio averaged over the entire area of the QC COC spotted standard resulted in a calculated concentration of 4100 ± 400 ng_{COC}/g_{tissue}, indicating that the MS/MS ion-signal ratios from the two tissues were comparable. This is important in quantitative MSI, as different tissue compositions, variations in tissue thickness, inconsistent matrix application, and uneven internal standard concentrations can all affect the detected ion signal. Four areas from the positive sample were integrated and compared to the calibration curve generated from the control tissue. This resulted in an average concentration of ~400 ± 50 ng_{COC}/g_{tissue}. Error in calculation of the spotted COC standard concentrations in tissue can also contribute to error in the determined COC concentration in the positive sample, as stated previously.

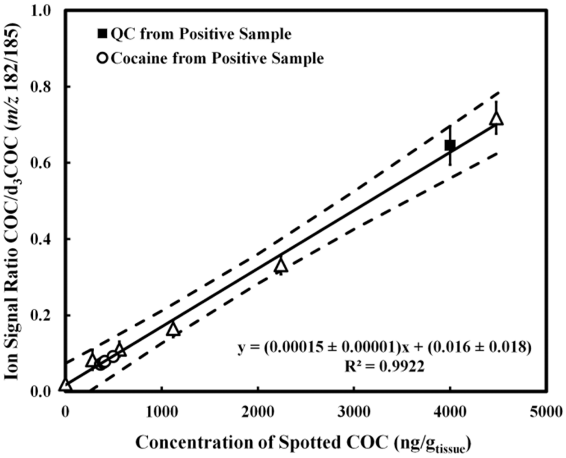


Figure 2. Normalized calibration plot produced with wide-isolation MS/MS on the Q-TOF instrument for COC spotted beneath a negative control section of brain tissue similar to adding calibration standards to control matrix for LC-MS quantitation. Product ion-signal ratio (*m*/*z* 182/185) from the positive sample was compared to the curve to estimate the concentration of COC. The QC sample served as a means to ensure that the signal between the two tissues was comparable. The calculated concentration of the QC sample from the calibration curve was within 5% of the spotted concentration. Error bars represent the standard errors from the variation in the ~40 MS/MS spectra averaged for each calibration point.

The MSI results from the Q-TOF instrument were compared to those from similar experiments performed on the LTQ instrument. The same sample preparation protocol was followed, and similar instrument settings were utilized, except that the isolation window on the ion trap was 6 Da wide and centered at *m*/*z* 305.5 Da, which allowed for trapping of the $[M + H]^+$ ions of both COC and COC-d_3 simultaneously. A sample of the resulting ratio image of COC to COC-d_3 (*m*/*z* 182/185) is shown in Figure 3A, and the resulting standard addition plot (Figure 3B) yielded a COC concentration of 550 ± 1000 ng/g_{tissue}. The large error in the 95% confidence interval, compared to that in Figure 1D, is due to the high signal variation and the error in back-extrapolation to the *x* axis. However, we report fairly good linearity (R^2 = 0.9853) from the spiked calibration standards. Analysis of three serial sections by standard addition from the MSI experiments with the LTQ instrument yielded an average concentration of 880 ± 300 ng/g_{tissue}

The MS/MS images from the LTQ instrument were similar to those obtained with the Q-TOF instrument; however, a significantly larger concentration of COC with larger uncertainty within each experiment was observed. The differences in the magnitude of the uncertainty between the

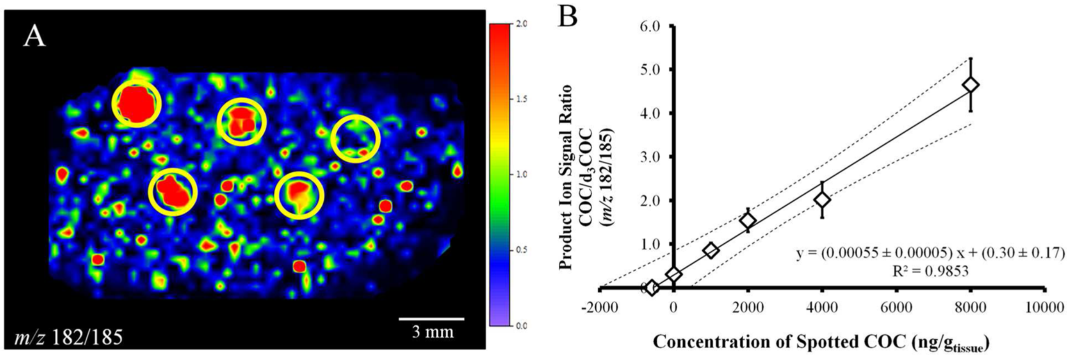


Figure 3. (A) Product ion ratio image generated from wide-isolation MS/MS from MSI experiment performed on the LTQ instrument. (B) Corresponding standard additions plot. From this wide-isolation MS/MS experiment, the concentration of COC was calculated to be 550 ng/g_{tissue}.

two instruments can be attributed primarily to the ion source resulting in significant differences in the amount of sample analyzed at each raster step. MSI analysis with the Q-TOF instrument was performed with a significantly more powerful 200-Hz, frequency-tripled Nd:YAG laser fired 200 times at each raster step. This resulted in nearly complete ablation of the 20-μm-thick sample at each raster position, as indicated by visual observation of the tissue. MSI analysis with the LTQ instrument was performed with a less powerful 60-Hz nitrogen laser fired only eight times at each raster step, resulting in no visible damage to the tissue after MSI analysis. This difference can significantly affect the precision of the measurements. Complete laser ablation through the entire tissue on the Q-TOF instrument results in larger sample consumption at each spot and, thereby, higher signal and better precision. Complete ablation of an area of tissue 150 μm in diameter and 20 μm thick (350000 μm^2) means that the mass of tissue ablated at each spot with the Q-TOF instrument was ~370 ng. Given that no visual damage to the tissue was observed after the LTQ experiments, perhaps only 1−10% of the material was ablated, leading to poorer precision. Laser energy fluctuations on the LTQ instrument might also contribute to the ion-signal variability, although this could be minimal, as the variance reported by the manufacturer is 4% from shot to shot. Further, employing multiple laser pulses per raster spot significantly reduces this effect.

One could anticipate that the internal standard (applied beneath the tissue) might not be completely evenly distributed through the thickness of the tissue following extraction through the tissue (into the wet tissue and/or with application of the MALDI matrix solution); thus, one could assume that the concentration profiles (through the tissue thickness) of the analyte (which was initially distributed uniformly) and the internal standard (which was initially just at the bottom of the tissue) would differ. Therefore, the analyte/internal standard ratio would be expected to vary across the tissue thickness, with the ratio higher near the top of the tissue and lower (i.e., more internal standard present) near the bottom of the tissue. If one ablated the tissue completely, as on the Q-TOF instrument, this variation in the distribution of analyte and internal standard would be negated, whereas if one ablated only the top 1% or 10% of the tissue, as on the LTQ instrument, then one would preferentially detect analyte instead of internal standard, making the measured analyte concentration higher than expected.

Summing 200 spectra with TOF MSI instruments is routinely performed to increase the signal-to-noise ratio and to obtain quality images. The LTQ instrument software does not permit 200 laser shots to fill the ion trap for a single spectrum, and even if it did, the time required to fill the trap given the 60-Hz laser repetition rate and the space charge limit of the trap would make this approach impractical. One could potentially obtain complete tissue ablation with the LTQ instrument by averaging 25−50 successive mass spectra, each with 4−8 laser shots, which would similarly increase the signal and signal-to-noise ratio. It is important to note that the LTQ instrument can generate excellent images with ablation of only a small fraction of the tissue at each spot (generally not possible with the Q-TOF instrument), but the ablation of only a small fraction of the tissue at each spot appears to reduce the precision of quantitative measurements and could lead to quantitative errors when using internal standard applied beneath the tissue. This issue is the focus of continuing research in our laboratory.

LC-MS/MS and MALDI Quantitation Using Tissue Extracts. Sections of the nucleus accumbens (~100 mg) were dissected and extracted according the procedures outlined previously. The extracts were analyzed by both MALDI wide-isolation MS/MS with scan-by-scan COC-to-COC-d_3 normalization and LC-MS/MS with a QqQ system. MALDI has been previously employed successfully to quantify small molecules;[14−17] however, the earlier experiments were not performed with scan-by-scan normalization of the analyte and internal standard signal. Employing wide-isolation MS/MS for the analysis of tissue extracts with scan-by-scan COC-to-COC-d_3 normalization on the LTQ instrument resulted in a significant improvement in signal reproducibility for each calibration standard. The average relative standard deviation (RSD) of the 50 MS/MS scans for the sample spots analyzed was 45% for the COC (*m*/*z* 182) ion intensity. By normalizing the COC intensity of each MS/MS scan by the COC-d_3 signal (*m*/*z* 185), the average RSD was reduced to 17% across the spotted tissue extracts. We have previously shown that this signal variation in both the COC and COC/COC-d_3 ion signal is primarily due to matrix crystal differences across the area of the sample being analyzed.[19]

Table 1 reports the analytical performance of MALDI wide-isolation MS/MS compared to that of LC-MS/MS of the same tissue extracts, as well as the quantitative values determined by MSI. It is informative to compare MALDI quantitative results (MSI and tissue extracts) to LC-MS/MS experiments with MRM from a QqQ system, as the latter is a well-characterized quantitative analytical platform. The calibration curves produced from the tissue extracts analyzed by MALDI MS/

Table 1. COC Concentrations Determined by Various Analytical Methods[a]

method	COC conc (ng/g_{tissue})	std dev of replicates (ng/g_{tissue})
LC-MS/MS of extracts[b]	295, 254	18, 14
MALDI of extracts (LTQ)[b]	315, 266	34, 26
standard additions (Q-TOF)	240	110
standard additions (LTQ)	880	300
external calibration (Q-TOF)	415	50

[a] Means and standard deviations calculated from the replicate experiments. MALDI analysis of the tissue extracts was performed in quadruplicate, whereas all other analyses were performed in triplicate. [b] Two values represent two sections of the brain tissue analyzed by LC-MS/MS and MALDI wide-isolation MS/MS with the LTQ instrument.

MS compared to LC-MS/MS were comparable, as shown in Figure 4. A nearly ideal slope (~1.0) was observed from the LC-MS/MS experiments, indicating a linear instrument response factor to increasing analyte concentration. The deviation of the slope from 1.0 with MALDI can be attributed to background signal from the matrix or interference compounds from the extracts, because no chromatographic method was used prior to MS analysis of the extracts. Another factor contributing to the nonunity slope with MALDI might be the varying matrix-to-analyte ratio (M/A) of the analyte within the calibration standard concentrations. Varying the M/A ratio has been shown to have nonlinear effects on ion signal across a broad range of concentrations.[29] This effect was evaluated by applying different amounts of matrix to the samples, as shown in Figure 5. The amount of matrix could not be precisely determined because of spray-coating; however, the time for spray-coating was doubled from 30 s to 1 min and doubled again to 2 min to increase the amount of DHB applied. This resulted in calibration-curve differences attributed to varying M/A ratio of the calibration standards of COC. There appears to be an optimal range of the M/A ratio to produce adequate quantitative results. Standardizing the matrix application with automated matrix addition instruments (e.g., an ink-jet printer or Bruker ImagePrep matrix deposition device) would allow for more reproducible matrix addition in a uniform manner. More importantly, when the best linear MALDI calibration curve was used, the COC concentrations from the tissue extracts obtained by MALDI-MS/MS were similar to the concentrations (within error) of COC from the brain samples determined by LC-MS/MS.

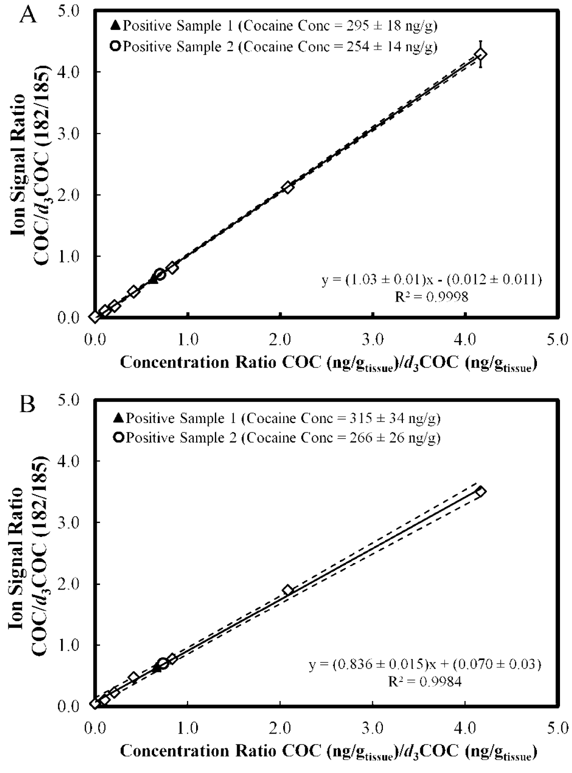


Figure 4. Calibration curves of the tissue extracts used to quantify COC in the nucleus accumbens determined by (A) LC-MS/MS and (B) wide-isolation MALDI MS/MS on the LTQ instrument. Analysis by MALDI MS/MS resulted in COC concentrations comparable to those obtained in the LC-MS/MS experiment.

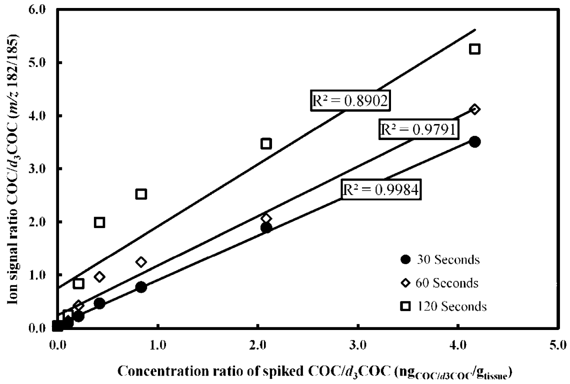


Figure 5. Calibration plots of the product ion-signal ratio with different amounts of DHB applied to the tissue extracts analyzed by wide-isolation MS/MS on the LTQ instrument. Because DHB was spray-coated, the amount of DHB applied was varied by time sprayed atop the spotted extracts. Nonlinear increases in the ratio of COC to COC-d_3 are observed with a nonideal M/A ratio.

Including spotting of the samples (two spots of 1 μL each) and quadruplicate MALDI-MS/MS analysis of each scan, the total experiment time was less than 1 h. Comparing this to LC-MS/MS experiments, with an LC gradient for each sample of 18 min, the total triplicate analysis time was ~13 h, including the analysis of solvent blanks to determine carryover. The total sample size for each MALDI analysis was 2 μL of the tissue extract compared to the 15 μL injection volume required for LC (triplicate analysis of 5 μL each). There is also a significant reduction in solvent use for MALDI analysis because the LC separation step is avoided. Improvements in MALDI sensitivity for the tissue extract could also be gained by reducing the reconstitution volume of the tissue extracts and by reducing the area of the spotted extract solution on the MALDI target, both of which would lead to a greater concentration of the analyte per unit area for analysis by MALDI.

The quantitative results from the MSI experiments are compared to the LC-MS/MS results for the tissue extracts in Table 1. The quantitative MSI results compare favorably to analysis by LC, specifically MSI quantitation by both standard additions and external calibration on the Q-TOF instrument (within 25% of the LC-MS/MS measured value). Quantitation by standard additions on the LTQ instrument resulted in a 3-

fold increase in the estimated COC concentration and relatively poor precision. As discussed in the section Quantitative Detection of COC by MS/MS Imaging, we attribute the poor quantitative performance of the LTQ instrument to incomplete laser ablation of the tissue in each spot and variations in the distributions of analyte and internal standard across the 20-μm thickness of the tissue. In contrast, the large number of laser shots at each spot employed with the Q-TOF system led to complete ablation of the tissue and thereby improved the precision and reduced the effects of variations in the depth profiles of the analyte and internal standard. This is reflected in the improved accuracy and reproducibility of the Q-TOF quantitative results; nevertheless, the absolute sensitivity of the LTQ system was superior, with excellent spectra and MSI images obtained with 25−50 times fewer laser shots.

The error in quantitative MSI results was found to be significantly larger, which is to be expected when performing direct tissue analysis with no sample cleanup. However, normalizing the wide-isolation MS/MS to the internal standard signal led to significant improvements in quantitative performance. These results show a significant improvement in quantitative MSI, which might lead to the use of the technique for relative or absolute quantitative determinations, particularly for whole-body imaging studies.

CONCLUSIONS

In this work, we have improved quantitative MSI by incorporating a uniformly applied internal standard combined with scan-by-scan COC-to-COC-d_3 ion-signal normalization. This approach overcomes the challenges associated with quantitative MALDI by accounting for ion-signal variability arising from either tissue heterogeneity, matrix crystal heterogeneity, or laser power fluctuations. Whereas tissue and crystal heterogeneity are most likely the main drivers of poor signal reproducibility, laser energy fluctuations could also be a source ion-signal variability; however, the use of multiple laser shots presumably reduces or eliminates issues of laser energy fluctuations. For MSI, employing an internal standard beneath a tissue sample for MSI not only improves quantitation but can also identify instances of tissue-specific ion suppression resulting from either poor analyte extraction or ionization resulting from different tissue types, as shown by Pirman et al.[28] This might lead to conclusive MS images providing valuable information about a particular compound's distribution in a specific organ or entire body without the need for further analysis. These techniques are applicable to numerous types of analysis and are not limited to MALDI imaging applications, but might be most useful in the area of pharmaceutical development specifically for determining the distribution of a new drug and its metabolites in a whole-body sample with the specificity advantages of MS. The development of quantitative imaging using other ionization techniques [e.g., secondary-ion mass spectrometry (SIMS), nanostructure-initiator mass spectrometry (NIMS), desorption/ionization on silicon (DIOS), desorption electrospray ionization (DESI)] might also benefit from the incorporation of a uniform internal standard, as tissue variability will likely be a main cause of ion-signal variability in these methods as well. With other ionization techniques, the same requirements to use the internal standard successfully still apply: The internal standard must by applied uniformly and must be detected in the same MS or MS/MS scan. Further development of quantitative MSI across multiple instrument platforms would allow the technique to be used universally for physiological and pharmaceutical determinations.

Use of this quantitative imaging approach does require some knowledge of the analyte concentration prior to the application of calibration standards and internal standards. This constraint can be addressed either by using data obtained from another method (e.g., LC-MS/MS) or by applying a wide range of calibration standards beneath subsequent tissue sections. Quantitation by MALDI either directly from tissue or from tissue extracts might also suffer from a limited dynamic range resulting from nonlinear matrix-to-analyte ratio effects inherent to MALDI. These limitations can make quantitative MALDI imaging challenging; however, working within the framework of the analytical capabilities of the technique can provide useful quantitative information. Employing an internal standard allows not only for quantitative MSI, but for quantitative MALDI analysis of tissue extracts as well. Analysis with MALDI reduced the analysis time from 13 to 1 h, a significant reduction in analysis time per sample. In some instances, MALDI with wide-isolation MS/MS could replace LC-MS/MS analysis with little reduction in analytical performance.

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Notes

The authors declare no competing financial interest.

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