Quantitative MALDI-MS^n Analysis of Cocaine in the Autopsied Brain of a Human Cocaine User Employing a Wide Isolation Window and Internal Standards

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Detection of drugs in tissue typically requires extensive sample preparation in which the tissue is first homogenized, followed by drug extraction, before the extracts are finally analyzed by LC/MS. Directly analyzing drugs in intact tissue would eliminate any complications introduced by sample pretreatment. A matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-MS^n) method as been developed for the quantification of cocaine present in postmortem brain tissue of a chronic human cocaine user. It is shown that tandem mass spectrometry (MS^2 and MS^3 increase selectivity, which is critical for differentiating analyte ions from background ions such as matrix clusters and endogenous compounds found in brain tissue. It is also shown that the use of internal standards corrects for signal variability during quantitative MALDI, which can be caused by inhomogeneous crystal formation, inconsistent sample preparation, and laser shot-to-shot variability. The MALDI-MS^n method developed allows for a single MS^3 experiment that uses a wide isolation window to isolate both analyte and internal standard target ions. This method is shown to provide improved precision [~10–20 times reduction in percent relative standard deviation (%RSD)] for quantitative analysis compared to using two alternating MS^3 experiments that separately isolate the target analyte and internal standard ions. (J Am Soc Mass Spectrom 2010, 21, 564–571) © 2010 American Society for Mass Spectrometry

Concentrations of drugs of abuse found in brain tissue better reflect drug concentrations at their site of action at the time of death than any other type of specimen used for postmortem forensic toxicology [1]. Conventional quantification of cocaine in brain tissue involves homogenate preparation, followed by extraction and/or derivatization. The extracts are then usually analyzed by gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS), GC, or LC. Lengthy extraction procedures are required to remove large concentrations of lipids and other endogenous materials present in the brain, which may interfere with the analysis. Multiple sample pretreatment steps also allow opportunity for loss of analyte, and tissue homogenization eliminates spatial information, which could provide histologically-specific drug distribution. Attempts have been made to determine the regional distribution of cocaine in postmortem brain of chronic human cocaine users [2–4]. These analyses were performed on sections of ~100–200 mg of tissue from different regions of the brain, which were assumed to be homogeneous and accurately representative of the drug concentration in that excised region.

Direct MALDI-MS analysis of intact tissue can provide quantitative information about the distribution of cocaine in human brain more rapidly, with higher spatial resolution, and with less sample loss than drug analysis methods that involve tissue homogenization. Furthermore, the distribution of cocaine in brain tissue acquired by MALDI-MS can be directly related to the histology. The majority of MALDI-MS instruments use a time-of-flight (TOF) mass analyzer, which has benefits of high mass range and high throughput. Quantitative MALDI-MS is challenging, however, because MALDI exhibits irreproducible signal intensities due to inhomogeneous crystal formation, inconsistent sample preparation, and laser shot-to-shot variability. A typical MALDI-TOF experiment will obtain 200–1000 consecutive mass spectra at each sample spot (one laser shot per spectrum), which are averaged to improve the reproducibility of the MALDI signal [5]. Similarly, MALDI-MS instruments that utilize a linear ion trap (LIT) mass analyzer can obtain multiple mass spectra at each spot and average them to improve reproducibility. Here we obtain a single mass spectrum at each spot, with typically 10 laser shots used to fill the ion trap for each spectrum. Note that any ion trap has a finite ion

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storage capacity before space-charge reduces resolution and causes peak shifts. MALDI-LIT instruments can minimize space-charge effects by utilizing automatic gain control (AGC), which automatically controls the number of laser shots used to fill the trap (typically 1–20 shots) to optimally fill the ion trap for maximum signal without loss of mass resolution. Laser power can also be optimized along with choice of matrix compound to maximize analyte signal while avoiding space-charge effects. The use of internal standards for quantitative MALDI-MS has been shown to improve signal stability, if the solution-phase properties are carefully matched as in an isotopic standard [6].

Quantification of small drug molecules like cocaine using MALDI-MS is further complicated by the presence of interfering matrix peaks in the low mass range along with ions that may be produced from endogenous compounds present in the brain tissue. One of the strengths of a linear ion trap mass spectrometer is its ability to perform multiple stages of mass analysis (MS^n) to significantly increase the selectivity for the analyte of interest. A MALDI-MS^n method could be developed to remove interferences from both MALDI matrix and the complex sample environment of brain tissue; however, a problem arises when trying to combine the use of MS^n with the use of internal standards. Instrument software allows for only one isolation window (IW) in MS^n experiments, isolating one parent mass (or range of masses) for collision-induced dissociation (CID). This means that MS^n of the target ions of the analyte and internal standard would typically be performed with two separate MS^n experiments. This would increase the response variability and could counteract the signal normalizing effects of using an internal standard. In contrast, using a 6-Dalton (Da)-wide IW centered at a mass-to-charge (m/z) between the $[M + H]^+$ ions of cocaine and its trideuterated analog allows for isolation and CID of both ions during a single MS^n experiment. This single isolation method reduces the signal variability inherent with MALDI compared to isolating each ion individually with a 1-Da IW (in two alternating MS^n experiments). This method is used here to detect and quantitatively image cocaine in postmortem human brain tissue.

This study demonstrates that MS^n increases selectivity, which is critical for differentiating analyte ions from matrix ions and endogenous compounds found in brain tissue. It is also shown that the use of internal standards corrects for signal variability in quantitative MALDI arising from inhomogeneous crystal formation, inconsistent sample preparation, and laser shot-to-shot variability. Using a single MS^n experiment with a wide IW to isolate both analyte and internal standard target ions provides improved precision (10–20 times reduction in %RSD) for quantitative imaging studies compared to using two alternating MS^n experiments that isolate the analyte and internal standard target ions separately.

Experimental

Chemicals

Cocaine (COC; MW 303.4 Da) and COC-d_3 (MW 306.4 Da, 0.29% d_o) were purchased from Cerilliant (Round Rock, TX, USA) at concentrations of 1 mg/mL and 100 μg/mL, respectively, in acetonitrile. High-performance liquid chromatography (HPLC)-grade acetonitrile, methanol, and water were purchased from Fisher Scientific (Pittsburgh, PA, USA). Working standards of COC and COC-d_3 were diluted with acetonitrile and then stored at 4 °C. COC calibration standards were prepared in acetonitrile at concentrations of 5.0, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.020, 0.010, and 0.005 μg/mL with the COC-d_3 internal standard at a concentration of 2.0 μg/mL. Sinapinic acid (SA; MW 224.2 Da), 2,5-dihydroxybenzoic acid (DHB; MW 154.1 Da), and α-cyano-4-hydroxycinnamic acid (CHCA; MW 189.2 Da) were purchased from Acros Organics (Geel, Belgium). Saturated matrix solutions (40 mg/mL DHB, 10 mg/mL SA, and 10 mg/mL CHCA) were prepared in methanol/water (70:30, vol/vol) on the day of use.

Tissue Collection

Human brain tissue samples were provided by the El Paso County Coroner's Office in Colorado Springs, CO. Postmortem brain material was excised from the nucleus accumbens (NAc) from case number 07A-369, whose toxicologic analysis indicated the presence of cocaine in blood at 69 ng/mL (COC concentration in the brain tissue was not quantified). The NAc is a dopamine-rich area of the striatum, which may contain an accumulation of COC due to its affinity to bind with the dopamine transporter [7]. At autopsy, the excised tissue was immediately snap-frozen in liquid nitrogen and then stored in a −80 °C freezer until analyzed.

Tissue Sectioning and Sample Preparation

Frozen brain tissue was cut into thin sections (20 μm thickness) in a cryostat (HM 505E; Microm International GmbH, Waldorf, Germany) at −25 °C. The tissue samples were frozen to the cryostat sample stage using distilled water. Serial brain sections were collected onto microscope slides where they were thaw mounted and then stored at −80 °C. Before mass spectrometric analysis, the tissue sections were removed from the freezer and placed in a vacuum desiccator for 30 min before spiking standards (1-μL droplets by micropipet) and applying MALDI matrix. The matrix was applied to the tissue sections using an artistic airbrush (Aztek A470; Testors, Rockford, IL, USA). The application of MALDI matrix by airbrush has been previously published [8]. Matrix was applied using the dried-droplet method for experiments performed on MALDI plate.

Mass Spectrometry

Mass spectra were acquired using an LTQ linear ion trap with a vMALDI ion source (Thermo Finnigan, San Jose, CA, USA), equipped with a nitrogen laser (337 nm) at a frequency of 20 Hz and 100-μm spot size. A more detailed description of this instrument has been published [8]. An average of 10 laser shots per scan was used to produce mass spectra, except for experiments that used AGC, in which the number of laser shots was automatically varied to optimally fill the trap with ions, thus avoiding space charge-related peak broadening and mass shifts. AGC assesses the ion generation rate by use of a prescan, and then adjusts the number of laser shots per scan to produce a similar number of ions for each scan. The spectra are normalized to the number of laser shots for each scan.

Resonance excitation is used for isolation, activation, and mass analysis. For MS^n experiments, unwanted ions are resonantly ejected from the ion trap by applying a 5–500 kHz multi-frequency isolation waveform consisting of sine components spaced every 0.5 kHz. The ions of interest are isolated by removing sine components from the isolation waveform that correspond to the secular frequency of the desired ion(s). Ions are selected for isolation in the LTQ software by entering the *m/z* with its IW. The mass range for the ion is defined as (*m/z* −IW/2) to (*m/z* +IW/2). The IW should be narrow enough to minimize including interfering peaks, but wide enough to avoid loss of sensitivity for the desired ion(s). However, it is important to note that the activation width for resonance excitation (CID) has the same value as the IW. Therefore, the collision energy applied during MS^n is spread over the activation width. Thus, increasing the IW decreases the true collision energy for each ion.

The tissue-mounted microscope slides were affixed to a slide holder plate with double-sided tape. The plate was then inserted into the LTQ, and the plate was rastered beneath the laser spot at 100-μm steps to produce position-specific mass spectra. Specific ions and the total ion current (TIC) signal were extracted from the raw data files using ImageQuest version 1.0 (Thermo Fisher Scientific, San Jose, CA, USA), which was used to generate an image.

Results and Discussion

MS^2 and MS^3 Mass Spectra of COC and COC-d_3

DHB was selected as the MALDI matrix in this study, as preliminary investigations showed that it produces more efficient ionization for COC at low concentrations than SA or CHCA. DHB was also preferred as the matrix for COC analysis due to its lack of interference with the $[M + H]^+$ ion of COC (*m/z* 304) and COC-d_3 (*m/z* 307). The COC standards in acetonitrile were characterized by MS^n. The MS^2 spectrum of *m/z* 304 and 307 (IW = 1.0 Da, CID = 20) each show one major product ion, corresponding to a neutral loss (NL) of benzoic acid (NL 122) at *m/z* 182 and 185, respectively. MS^3 was performed on the product ion signal at *m/z* 182 of COC (IW = 1.0 Da; CID = 30), resulting in product ions at *m/z* 150 (NL of 32; CH_3OH), *m/z* 122 (NL of 60; CH_3OH + CO), *m/z* 119 (NL of 63; $CH_3OH + CH_3NH_2$), *m/z* 108 (NL of 74; $CH_3OH + CH_2CO$), *m/z* 91 (NL of 91; $CH_3OH + CH_3NH_2$ + CO), and *m/z* 82 (NL of 100; $CH_3OH + C_4H_4O$ via a 6-electron Alder ene rearrangement). The structures of the fragment ions of the $[M + H]^+$ ion of COC and its proposed fragmentation pathway have been previously published [9]. MS^3 was performed on the product ion signal at *m/z* 185 of COC-d_3 (IW = 1.0 Da; CID = 30) resulting in product ions at *m/z* 153 (NL of 32; CH_3OH), *m/z* 125 (NL of 60; CH_3OH + CO), *m/z* 119 (NL of 63; $CH_3OH + CH_3NH_2$), *m/z* 111 (NL of 74; $CH_3OH + CH_2CO$), *m/z* 91 (NL of 91; $CH_3OH + CH_3NH_2$ + CO), and *m/z* 85 (NL of 100; $CH_3OH + C_4H_4O$). The *m/z* values of the fragment ions of COC-d_3 at *m/z* 91 and 119 are the same as those for COC because these ions have lost the trideuterated tag that was originally located on the *N*-methyl group.

Improving Signal Reproducibility with Internal Standards

Quantitative analysis by MALDI is challenging, because of signal irreproducibility due to variation in sample preparation, inhomogeneous co-crystallization of analyte and MALDI matrix, and laser shot-to-shot variability. Table 1 shows the mean *m/z* 304 signal of the $[M + H]^+$ ion of COC detected from COC/COC-d_3 standard solutions spotted 1 μL each in triplicate onto a MALDI plate with 1 μL of DHB matrix pipetted on top. The COC/COC-d_3 solutions were composed of different concentrations of COC (5.0, 2.5, 1.2, and 0.63 μg/mL) mixed with 1.0 μg/mL of COC-d_3. The left side of Table 1 shows the high variability in signal for each concentration with %RSD ranging from 29 to 67%, making it difficult to distinguish signal from one concentration to another. The right side of Table 1 shows the *m/z* 304 signal of COC normalized to the $[M + H]^+$ ion signal of COC-d_3 at *m/z* 307. Signal variability was reduced dramatically (%RSD ranged from 0.27 to 1.33%) by normalizing the analyte signal to that of the internal standard making quantification by MALDI possible.

Table 1. Signal of *m/z* 304, $[M + H]^+$ of COC and *m/z* 304 signal ratioed to *m/z* 307 signal $[M + H]^+$ of COC-d_3

Cocaine Conc (μg/mL)	*m/z* 304		*m/z* 304/307	
	Mean	% RSD	Mean	% RSD
0.63	6.0E + 05	61	0.61	1.33
1.25	1.8E + 06	29	1.23	1.20
2.50	2.4E + 06	67	2.57	0.27
5.00	1.9E + 06	32	5.21	0.59

All solutions spotted 1 μL in triplicate on MALDI plate with DHB matrix. The internal standard (COC-d_3) was maintained at 1 μg/mL for all solutions.

Increasing Analyte Selectivity with MS^n

Figure 1a shows a full-scan MS spectrum of a 1.25:1 mixture (by mass) of COC and COC-d_3 standards spiked (1 μL of 1.25 μg/mL and 1.0 μg/mL, respectively) onto a MALDI plate with DHB matrix. Peaks at *m/z* 304 and 307 represent the $[M + H]^+$ ions of COC and COC-d_3, respectively. A number of cluster ions, fragment ions, and a molecular ion of DHB are also present, including *m/z* 137 $[DHB + H - H_2O]^+$, *m/z* 154 $[DHB]^+$, *m/z* 177 $[DHB + Na]^+$, *m/z* 199 $[2DHB + Na]^+$, *m/z* 221 $[DHB - 2H + 3Na]^+$, *m/z* 273 $[2DHB + H - 2H_2O]^+$, *m/z* 291 $[2DHB + H - H_2O]^+$, and *m/z* 331 $[2DHB + Na]^+$. Figure 1b shows a full-scan MS spectrum of a 1:1 mixture (by mass) of COC and COC-d_3 standards spiked (1 μL of 1.0 μg/mL each) onto a 20-μm thick human brain tissue slice with DHB airbrushed. The $[M + H]^+$ ions of COC and COC-d_3 are observed at *m/z* 304 and 307, respectively. The same cluster ions, fragment ions, and molecular ion of DHB are present, in addition to numerous ions of endogenous compounds from the brain tissue, including the phosphocholine head group of phosphatidyl choline at *m/z* 184 $[(CH_3)_3NCH_2CH_2PO_4H]^+$. Identification of COC and COC-d_3 on the MALDI plate and brain tissue was confirmed by characteristic MS^2 product ions at *m/z* 182 and 185, respectively.

MS^2 spectra of *m/z* 304 with COC spiked at concentrations below 5 ng/mL on plate and on tissue revealed an isobaric compound that has product ions at *m/z* 212 and 91. The isobaric ion likely originates from the surfactant benzyldimethyldodecylammonium chloride (C_{12}BAC) [10]. The widespread use of C_{12}BAC and other BACs as disinfectants makes it a likely trace contaminant in the laboratory. The ion at *m/z* 212 results from fragmentation of the carbon–nitrogen bond between the toluyl substituent and the quaternary amine. The *m/z* 91 ions is a stable tropylium ion formed by fragmentation in which the toluyl substituent retains the positive charge. MS^2 of *m/z* 304 with COC spiked at concentrations below 5 ng/mL also results in the detection of product ions of isobaric compounds at *m/z* 256 and 286. These ions have not yet been identified, but are not present when DHB has been characterized on MALDI plate alone.

The presence of isobaric ions in samples increases with sample complexity and may interfere with quantification at low analyte concentrations. MS^n can improve analyte selectivity and produce higher signal-to-noise ratios, resulting in lower detection and quantification limits for the analyte. Combining the use of MS^n with internal standards is commonly performed by alternating MS^n scans of the analyte and the internal standard ions, and then ratioing the resulting product ion signals. This method is effective for use with ionization techniques such as electrospray and atmospheric pressure chemical ionization; however, due to

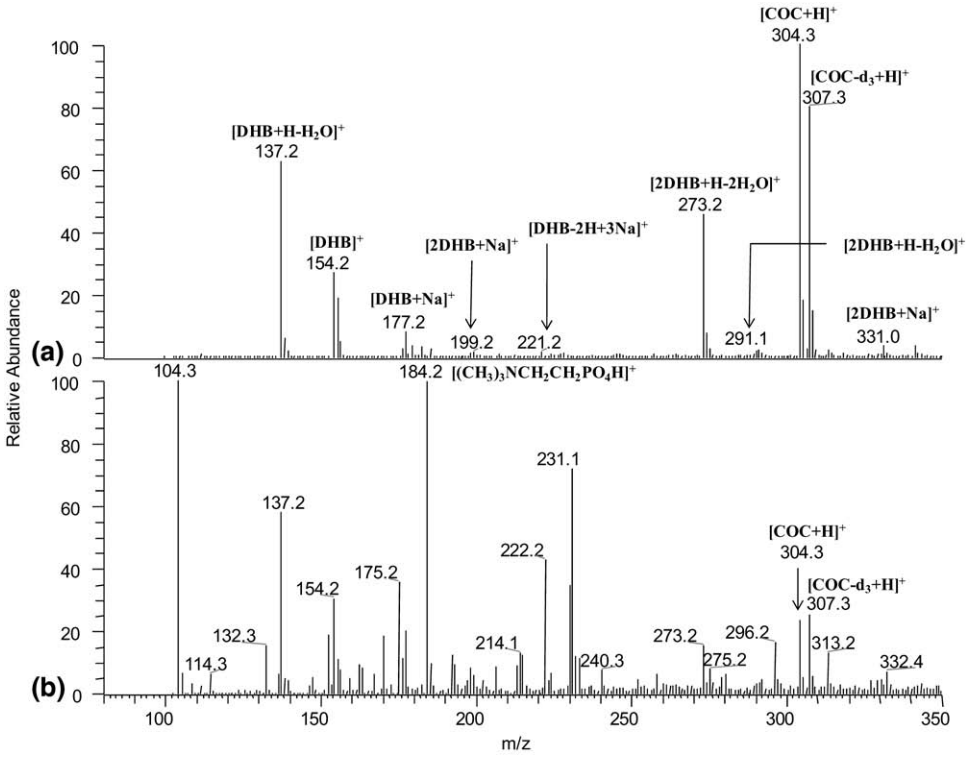


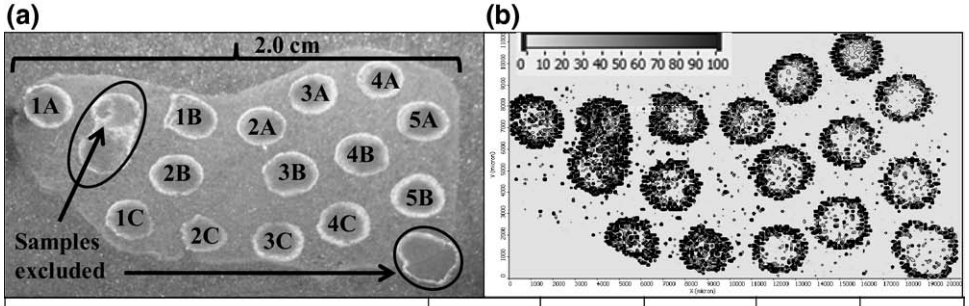
Figure 1. MALDI mass spectrum of (**a**) a solution of COC (1.25 μg/mL) and COC-d_3 (1.0 μg/mL) spotted (1 μL) with DHB matrix on MALDI plate and (**b**) a solution of COC and COC-d_3 (1.0 μg/mL each) spiked (1 μL) on postmortem human brain tissue with DHB matrix airbrushed.

the shot-to-shot variability of MALDI, acquiring analyte and internal standard signals in alternating MS^n experiments may counteract the signal normalizing effects gained by using an internal standard.

Combining Internal Standards with MS^n Using a Wide Isolation Window

One method for combining the use of internal standards with MS^n is to perform MS^2 on the analyte and internal standard ions separately during alternate MS^n experiments. The $[M + H]^+$ ion of COC (*m/z* 304) is isolated with a 1 Da window and then collisionally activated to produce the product ion at *m/z* 182. In a separate MS^2 scan, the $[M + H]^+$ ion of COC-d_3 (*m/z* 307) is isolated with a 1 Da window and CID is applied, resulting in the product ion at *m/z* 185. The analyte ion signal at *m/z* 182 can then be normalized to the internal standard ion signal at *m/z* 185. An alternative approach to using two separate MS^n experiments is to use a single wide isolation window (e.g., 6 Da) centered at *m/z* 305.8 allowing the simultaneous isolation and CID of the $[M + H]^+$ ion of COC (*m/z* 304) and COC-d_3 (*m/z* 307). The resulting MS^2 spectrum contains the product ions of COC and COC-d_3 at *m/z* 182 and 185, respectively.

The performance of the MS^n experiment using a single wide isolation window was compared with that using two alternating MS^n experiments by detecting COC and COC-d_3 spiked on top of human brain tissue. Figure 2a shows a microscope image of a 20-μm thick human brain tissue slice with COC/COC-d_3 solutions spotted 1 μL each in triplicate (A, B, and C) on the surface of the tissue and then airbrushed with DHB. The five COC/COC-d_3 solutions spotted all contained 2.0 μg/mL of COC-d_3 in addition to 0.31, 0.62, 1.2, and 5.0 μg/mL of COC, respectively. The compositions of the solutions spotted (1–5) are shown in the table below the image. The average dried spot size was 0.25 cm in diameter. Figure 2b shows the MS^2 product ion image of *m/z* 305.8 (IW = 6 Da, CID = 20) of the entire tissue slice generated from signal extracted from the mass range *m/z* 182-186 and normalized to the TIC. Higher signal intensity correlates with the darker shade of gray, illustrating how the COC and COC-d_3 co-crystallize along with the DHB towards the edge of each spot. The LTQ software was used to outline each spot to be analyzed. Each spot was analyzed twice: first by performing MS^2 of *m/z* 304 (IW = 1 Da, CID = 20) followed by MS^2 of *m/z* 307 (IW = 1 Da, CID = 20), and then by MS^2 of *m/z* 305.8 (IW = 6 Da, CID = 20). For each analysis, all of the spectra (~500 scans) were averaged for each spot, and the *m/z* 182 signal for COC was normalized to the *m/z* 185 signal for COC-d_3 and plotted against the concentration of COC spiked to produce two different calibration curves. The first calibration curve was the average ratio of peak intensities *m/z* 182 to 185 as a function of the spiked COC concentration for the alternating MS^2 experiment (i.e., MS^2 of *m/z* 304 in one scan and then MS^2 of *m/z* 307 the following scan). The line of best fit was $y = 0.68(\pm0.07)x + 0.2(\pm0.2)$ over the range 0.31 to 5.0 μg/mL with a standard error of the estimate (SEE) = 0.2833; the %RSD ranged from 12% to 30%. The 95% confidence intervals for the slope and y-intercept were 0.44 to 0.91 and −0.4 to 0.9, respectively. The second calibration curve was the average ratio of peak intensities *m/z* 182 to 185 as a function of the spiked COC concentration for a single MS^2 experiment with a wide 6-Da isolation window centered at *m/z* 305.8 (i.e., MS^2 of *m/z* 304 and *m/z* 307 in one scan). The line of best fit was $y = 0.492(\pm0.001)x + 0.023(\pm0.003)$ over the range 0.31 to 5.0 μg/mL with an SEE = 0.0052; the %RSD ranged from 0.50% to 5.1%. The 95% confidence intervals for the slope and y-intercept were 0.488 to 0.496 and 0.011 to 0.034, respectively. Precision was dramatically improved by using the single MS^2 experiment with 6-Da wide isolation window compared with isolating each ion individually with a 1-Da window (two alternating MS^2 experiments).



Solution # (Triplicate Runs A, B, C)	1	2	3	4	5
COC (μg/mL)	5.0	2.5	1.3	0.62	0.31
COC-d_3 (μg/mL)	2.0	2.0	2.0	2.0	2.0

Figure 2. (**a**) Photomicrograph of 20 μm thick human brain tissue mounted on slide with COC/COC-d_3 solutions spiked (1 μL) in triplicate (A, B, and C) on top of tissue and then airbrushed with DHB matrix. (**b**) MS^2 product ion image generated from signal selected from mass range *m/z* 182–186 and normalized by the TIC.

There was a 10–20 times reduction in %RSD and a 50 times reduction in SEE by using the wide isolation method. Supplementary material can be found in the electronic version of this article.

Isolation Window Width and Automatic Gain Control

Usually the smallest isolation width is desired for MS^n experiments performed with an ion trap mass spectrometer to avoid isolating unwanted background ions and reducing analytical specificity. The minimum acceptable ion isolation width is defined as the lowest range providing no appreciable signal attenuation of the analyte and internal standard ions when compared to a wider setting. Signal attenuation can result either from losses during the resonance ejection step which is used to remove masses below and above the selected *m/z* range, or from decreased CID efficiency of the analyte and internal standard ions.

The effect of isolation width on the intensity of the product ions of the $[M + H]^+$ ions of COC (*m/z* 304) and COC-d_3 (*m/z* 307) together in a single MS^2 scan was investigated. Five solutions of COC and COC-d_3 were prepared at equal concentrations and diluted with acetonitrile (0.12, 0.25, 0.50, 1.0, and 2.0 μg/mL). All five solutions were spotted in triplicate 1 μL each onto a MALDI plate followed by 1 μL of DHB matrix. For all MS^2 experiments, the parent ion was set to *m/z* 305.8, the center of the mass range between *m/z* 304.3 and 307.3, and the CID was set to 20. The size of the isolation window width centered at *m/z* 305.8 was varied (4, 6, and 8 Da), and the ratio of the intensities of the products ions at *m/z* 182 and 185 for COC and COC-d_3, respectively, were observed. It is important to note that MS^2 of *m/z* 304 (IW = 1.5 Da) produced a negligible amount of *m/z* 185 (<0.0005%), the product ion of *m/z* 307. Also, MS^2 of *m/z* 307 (IW = 1.5 Da) produced a negligible amount of *m/z* 182 (<0.002%), the product ion of *m/z* 304. The expected signal ratio of COC to COC-d_3 is 1.02 for equal masses based on a calculated molar ratio of 1.01 corrected for the isotopic purity of COC-d_3 (0.29% COC-d_0). The measured signal ratio of *m/z* 182 to 185 was approximately equal to 1 for concentrations below 0.50 μg/mL, but the ratio increased at concentrations above 0.50 μg/mL (i.e., the *m/z* 185 signal decreased with respect to *m/z* 182). It was also observed that the signal ratio of *m/z* 182 to 185 was higher for a 4 Da isolation window (2.07 at 1.0 μg/mL and 3.50 at 2.0 μg/mL) compared with the 6 Da (1.18 at 1.0 μg/mL and 1.68 at 2.0 μg/mL) and 8 Da (1.05 at 1.0 μg/mL and 1.81 at 2.0 μg/mL) isolation windows widths. This suggests that either some of the *m/z* 307 ion is being lost during isolation or that the *m/z* 307 ion is being less efficiently excited during the CID step when narrower IWs are used.

An effort was made to separate the isolation step from the CID step of the MS^2 experiment to better understand the effect of isolation window width on the signal intensities of the MS^2 product ions of the $[M + H]^+$ ions of COC and COC-d_3. The above experiment was repeated on the five COC/COC-d_3 solutions, except that no CID voltage was applied so that the ions at *m/z* 304 and 307 were isolated but not fragmented. The ratio of intensities of *m/z* 304 to 307 was then monitored for different isolation window widths (4, 6, and 8 Da). Results showed that the signal ratio of *m/z* 304 to 307 remained approximately equal to 1 for concentrations 0.12–2.0 μg/mL for isolation widths of 6 and 8 Da; however, the signal ratio steadily increased for a 4 Da isolation window at concentrations above 0.50 μg/mL. The increase in the signal ratio of *m/z* 304 to 307 (i.e., *m/z* 307 signal decreased with respect to *m/z* 304) at higher concentration is presumably due to a mass shift of *m/z* 307 outside the isolation window, resulting in resonance ejection of some of the *m/z* 307 ions. This mass shift could be caused by space-charge effects at higher ion populations in the ion trap, and may be corrected by using AGC. The experiment was repeated again, comparing the signal ratio of *m/z* 304 to 307 with and without AGC with a 4 Da isolation window and no CID applied. Results showed that when AGC was used, the signal ratio of *m/z* 304 to 307 remained approximately equal to 1 for all concentrations analyzed (0.12–2.0 μg/mL), indicating that AGC can minimize space-charge effects, which may lead to ejection of the higher *m/z* ion when a narrower isolation width is employed.

Quantification of Cocaine in Postmortem Human Brain Tissue

The MS^2 wide isolation method developed for COC was applied to human brain tissue from a subject whose toxicology report showed the presence of COC. The MS^2 product ion of the $[M + H]^+$ ion of COC at *m/z* 182 was not distinguishable from the background signal; therefore, an MS^3 wide isolation method was developed to increase selectivity. The MS^3 wide isolation method was evaluated by spotting 1 μL of a 4.0 μg/mL solution of COC and COC-d_3 onto a MALDI plate followed by 1 μL of DHB matrix. The method involves centering a 6-Da isolation window at *m/z* 305.8 and applying a CID of 20 followed by a 6-Da isolation window centered at *m/z* 183.5 (between COC and COC-d_3 product ions at *m/z* 182 and 185) with a CID of 30. The resulting MS^3 product ion spectrum revealed characteristic fragment ions of COC at *m/z* 150, 82, 108, 122, 119, and 91 and for COC-d_3 ions at *m/z* 153, 85, 111, 125, 119, and 91. The MS^3 wide isolation method was applied to unspiked brain tissue from a cocaine user, and COC was detected and confirmed by matching all six of these MS^3 ions. The relative intensities of the five most intense fragment ions (all but *m/z* 91) were within 12% of the standard fragment ion intensities.

Before quantifying unspiked COC in human brain tissue with the MS^3 method, it was necessary to show

that the response factors for COC and COC-d_3 were equal, so that the calibration curve of COC-d_3 could be used. A series of 1:1 solutions of COC and COC-d_3 at various concentrations (0.03, 0.06, 0.13, 0.25, 5.0, 1.0, and 2.0 μg/mL) were prepared and spiked in triplicate, 1 μL each, on top of serial tissue sections, and then DHB matrix was airbrushed over the tissue slices. Each spot was analyzed using the MS^3 wide isolation method, and the *m/z* 150 signal from COC was plotted versus the *m/z* 153 signal from COC-d_3. The slope of the plot was 1.062 $\pm$ 0.002 with a correlation coefficient $r^2 = 0.99{,}998$ over the concentration range 0.03 to 2.0 μg/mL. The 95% confidence interval for the slope was 1.057 to 1.066. The expected slope based on a molar ratio of 1.01 and an isotopic purity for COC-d_3 of 0.29% d_0 is 1.02, which means that COC has a 4% higher response factor than COC-d_3 over the concentration range measured.

The MS^3 wide isolation method was used to quantify the unspiked COC that was detected in the postmortem human brain tissue. Three different concentrations of COC-d_3 (0.06, 0.13, and 0.25 μg/mL) were spiked (1 μL) onto a glass slide before thaw mounting a 20 μm-thick brain tissue slice on top and airbrushing DHB matrix. All three spots were then analyzed using the MS^3 wide isolation method. Approximately 2000 scans were acquired to image the entire area of each of the spots (average area = 0.17 cm^2). The *m/z* 153 signal from each spot was used to develop a calibration curve that resulted in a line of best fit of $y = 399(\pm 27)x - 17(\pm 4)$. COC-$d_3$ was shown to have a linear response with increasing concentrations spiked underneath tissue. Since the MS^3 wide isolation method analyzes both COC and COC-d_3 simultaneously, unspiked COC was detected from each spot analyzed at *m/z* 150. An area of the tissue (500 MS scans) that was not spiked with COC-d_3 was analyzed using the MS^3 wide isolation method and the acquired *m/z* 150 signal was averaged with the *m/z* 150 signals from the spiked COC-d_3 spots, resulting in a very trace signal of 29 $\pm$ 1 counts. Assuming that the amount of unspiked COC extracted from the tissue has a 1:1 response with the COC-d_3 spiked on top of tissue, the calibration curve for COC-d_3 can be used to quantify the amount of COC present in the analyzed tissue. From the equation of the line, it was determined that COC was present at a level equivalent to 0.12 $\pm$ 0.01 μg/mL.

Using the 1 μL volume of COC-d_3 spiked underneath tissue, it is calculated that the mass of COC present is 1.2×10^{-4} μg. Given that the area of an analyzed spot on tissue was 0.17 cm^2 and that the tissue thickness was 20 μm (2.0×10^{-3} cm), the volume of tissue from which COC was extracted was 3.4×10^{-4} cm^3. The mass of the tissue is 3.4×10^{-4} g (density of wet tissue ~1.0 g/cm^3), resulting in an absolute concentration of COC detected in this area of the postmortem brain tissue of 0.35 μg/g (350 ppb).

The MALDI-MS method has a smaller sample requirement (~100 μg tissue) and less sample preparation than conventional GC/MS techniques, which require 1000 to 10,000 times more sample (0.1 to 1.0 g of brain tissue) to be homogenized before solid-phase extraction and GC/MS analysis [2, 3]. The GC/MS method developed by Kalasinsky et al. [2] reported a limit of detection of 0.1 ng/mL for the analysis of COC in brain tissue. COC was detectable at 30 ng/mL with the MALDI-MS^3 wide isolation method developed here. Although the MALDI-MS^3 wide isolation method is not as sensitive as the GC/MS method (primarily because it uses a 1000 times smaller sample), it readily detects cocaine at a level an order of magnitude below the lowest level (300 ng/mL) reported for COC detected by GC/MS analysis of 15 autopsied brain regions of 14 human chronic cocaine users [2].

Conclusions

It has been demonstrated that MS^2 and MS^3 increase selectivity, which is critical for differentiating analyte and internal standard ions from matrix ions and endogenous compounds found in brain tissue. It has also been shown that the use of internal standards corrects for signal variability during quantitative MALDI. A method was developed that allows for a single MS^2 experiment that uses a wide isolation window to isolate both analyte and internal standard ions. This method was shown to provide improved precision (~10–20 times reduction in %RSD) for quantitative analysis of COC in postmortem brain tissue compared with using two alternating MS^2 experiments that isolate the analyte and internal standard target ions separately. When COC concentration is too low to distinguish the MS^2 product ion at *m/z* 182 from the background, the MS^3 wide isolation method can be applied to increase selectivity.

The wide isolation window developed for the analysis of COC could be applied to quantitative MALDI-MS^n imaging of other drugs of abuse and their metabolites in brain tissue, which could prove to be an invaluable tool in the field of postmortem forensic toxicology. A MALDI-MS imaging method that combined the use of internal standards for minimizing signal variability with the high molecular specificity of MS^n could even provide a visual snapshot for the forensic toxicologist that reflects the true distribution and concentration of drugs of abuse at the time of death. This information could be used to substantiate fatal overdoses as well as provide supportive data for neurotoxicity studies.

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Appendix A
Supplementary Material

Supplementary material associated with this article may be found in the online version at doi:10.1016/j.jasms.2009.12.014.

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