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Technical Notes

Identification of Native Flavin Adducts from *Fusarium oxysporum* Using Accurate Mass Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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Accurate mass matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry is used to determine the identity of a naturally occurring substituted flavin adenine dinucleotide (FAD) of nitroalkane oxidase isolated from *Fusarium oxysporum*. Accurate mass measurements (1−2 ppm) combined with an elemental composition search identifies the unknown FAD substituent as nitrobutane. The use of internal and external calibration in accurate mass MALDI-TOF is discussed.

The use of electron impact (EI) ionization high-resolution mass spectrometry (HRMS) to determine the elemental composition of unknown compounds based on accurate mass measurements is a well-established analytical technique.[1] However, the use of HRMS in combination with chemical ionization (CI) and fast-atom bombardment (FAB) ionization is more difficult owing to the requirement for use of an internal calibrant compound to be mixed with the analyte for accurate mass calibration, in lieu of the gases used for calibration with EI HRMS. Calibration standards for FAB must have ions that bracket the mass-to-charge ratio (m/z) of the analyte ion and must also be compatible with FAB matrixes used for the analyte.[2] In addition it is important that the calibrant not suppress the ionization of the analyte.[3] These same factors must be considered when designing matrix-assisted laser desorption/ionization[4] (MALDI) HRMS experiments for accurate mass measurements. Wu et al.[5] recently reported the use of accurate mass MALDI Fourier transform ion cyclotron resonance (FTICR) HRMS in the identification of new compounds; here we illustrate the use of MALDI time-of-flight (TOF) HRMS for the determination of elemental composition using internal and external calibration.

MALDI-TOF MS is widely used in the analysis of peptides, proteins, and oligonucleotides, but the low mass resolution associated with this technique limits the mass measurement accuracy to 0.1−0.01%.[6] The recent introduction of delayed extraction (DE) or Wiley and McLaren's time-lag focusing[7] to MALDI-TOF greatly improves mass resolution[8−13] and mass measurement accuracy of both linear TOF[10,14] and reflectron (RTOF) mass spectrometers. For instance, MALDI-DE/RTOF mass resolution exceeds 10 000 ($m/\Delta m$)[11,15−16] and mass measurement accuracy is better than 5 ppm.[15−16] In this paper, we report the first use of accurate mass MALDI-TOF HRMS in determining elemental composition. Using mass spectrometry to determine the elemental composition of a compound larger than a few hundred daltons is difficult if not impossible because the mass measurement accuracy required to unambiguously determine elemental composition increases exponentially as the mass of the analyte increases.[17] However, when additional information is available about the unknown to be analyzed, specifically an unknown substituent on an otherwise well-characterized molecule, it is possible to determine the mass of the unknown substituent, by (mass of unknown) − (mass of known) = mass of substituent.[17] The compound analyzed in this study is a substituted flavin adenine dinucleotide (FAD), the native flavin adduct (R-FADH) (Figure 1) of nitroalkane oxidase from *Fusarium oxysporum*.[18]

MALDI-DE/RTOF mass spectra were acquired using a Voyager Elite XL mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with delayed extraction. The instrument has

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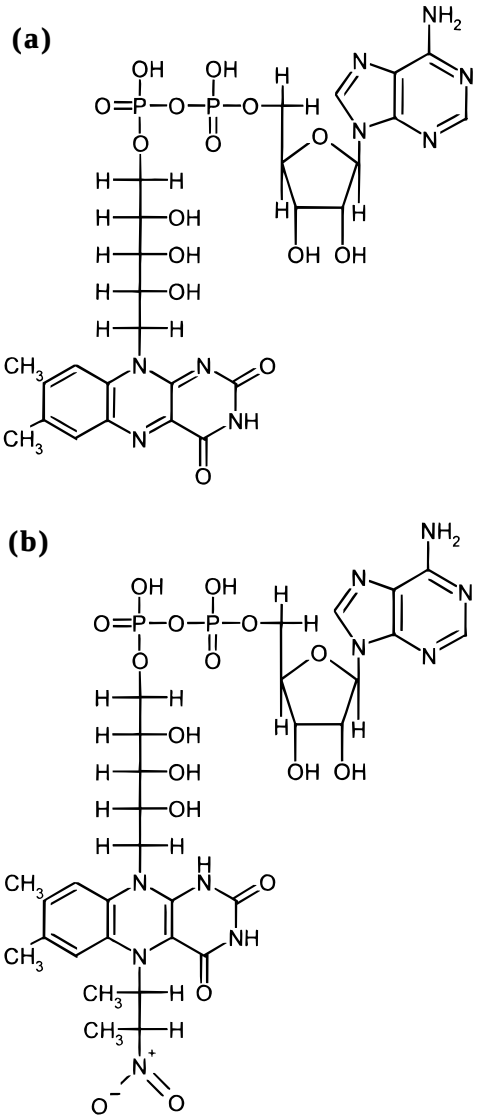

Figure 1. (a) Structure of flavin adenine dinucleotide (FAD). (b) Structure of the substituted flavin adenine dinucleotide (R-FADH) of nitroalkane oxidase from *F. oxysporum.* The proposed R group at the N(5) position is nitrobutane; also note the addition of a hydrogen at the N(1) position.

been described in detail elsewhere.[11,15] The negative ion mass spectra contained herein were acquired with an acceleration voltage of −20 kV, a pulse voltage of −5 kV, and a delay time of 250 ns. MALDI mass spectra of both the commercially available FAD (Sigma Chemical Co., St. Louis, MO) and the isolated R-FADH were acquired in the negative ion mode at a mass resolution ($m/\Delta m$) of ∼5500−6500. The R-FADH was isolated from *F. oxysporum* as described by Gadda et al.[18] Sample spots for FAD and R-FADH were prepared using the overlayer sample preparation method.[15] Briefly, 1 μL of a 0.15 M α-cyano-4-hydroxycinnamic acid (ACHA) (Sigma Chemical Co.) solution in MeOH was used to form a thin film of matrix crystals. A matrix/analyte solution was then prepared in a 2:1 H_2O/MeOH solution having final matrix/analyte concentrations of 10 mM and 2 μM, respectively. Then, 0.5 μL of the matrix/analyte solution was applied to the dried matrix crystals. Five spectrum were acquired from the sample spots, each spectra the average of 50 laser shots. A mass spectrum was acquired from a separate sample spot containing a mass calibration standard, α-melanocyte stimulating hormone (α-MSH) (M_r = 1663.7929). A calibration curve was generated using the α-MSH $[M - H]^-$ ion and the matrix dimer $[2M - H]^-$ ion by using the standard TOF equation:

$$\text{flight time } (t) = k_1(m/z)^{1/2} + k_2$$

This calibration curve was then applied to the mass spectra of the FAD and R-FADH (Table 1). Mass assignments were made using the all-^{12}C isotope peak and the peak centroid was determined using the top 50% of the peak as described by Edmondson and Russell.[15,16] The R-FADH was also analyzed using an internal standard to improve the mass measurement accuracy. A sample spot was prepared as described above with the matrix/analyte solution having final analyte concentrations of 4 and 1 μM for R-FADH and des-Arg^1-bradykinin (M_r = 903.4603), respectively.

Ten spectra (see Figure 2) were acquired and calibrated using the des-Arg^1-bradykinin $[M - H]^-$ ion and the matrix dimer $[2M - H]^-$ ion (Table 2). The first inset in Figure 2 contains an expanded region showing the $[M - H]^-$ ions of R-FADH at m/z 887, des-Arg^1-bradykinin at m/z 902, and a metastable fragment ion (loss of 30 from serine) of des-Arg^1-bradykinin at m/z 873.[19] The second inset further expands the $[M - H]^-$ ion region of R-FADH, revealing mass resolution of ∼6300. This mass resolution corresponds to a peak width (fwhm) of ∼8 ns, which is typical for ions observed using our instrument in the negative ion mode. We also typically observe partially unresolved detector rings in the negative ion mode, as illustrated in the second inset of Figure 2. The majority of the intense peak at m/z 784 corresponds to the $[M - H]^-$ ion of FAD, a prompt fragment ion of the R-FADH formed during the desorption/ionization event; a smaller portion of the peak corresponds to the metastable FAD $[M - H]^-$ ion.

A list was compiled for all elemental compositions having a m/z of 887.2143 ± 0.0044 (a ±5 ppm window). (Molecular Fragment Calculator 1.0 http://ram.chem.tulane.edu:8080/∼agroup/chemsoft/mfcalc.zip) The list contained over 500 possibilities. A second list was compiled using the mass of the adduct (R) of the R-FADH. This mass was obtained by subtracting the calculated m/z of the FAD $[M - H]^-$ ion plus a hydrogen from the m/z of the R-FADH $[M - H]^-$ ion (887.2143 (±0.00 68) − (784.149 32 + 1.007 825) = 102.0572 ± 0.0068. The list of possible elemental compositions for mass 102.0572 ± 0.0068 are contained in Table 3. Of the six possibilities, five can be eliminated on the

Table 1. Mass Measurement Accuracy of MALDI-DE/rTOF Using External Calibration

	m/z	
spectrum no.	FAD	R-FADH
1	784.1439	887.2207
2	784.1498	887.2099
3	784.1591	887.2194
4	784.1565	887.2186
5	784.1405	887.2030
av obsd	784.1500	887.2143
std dev (Da)	0.0071	0.0068
calcd	784.14932	887.21264
m/z error (Da)	0.0007	0.0017
m/z error (ppm)	0.9	1.9

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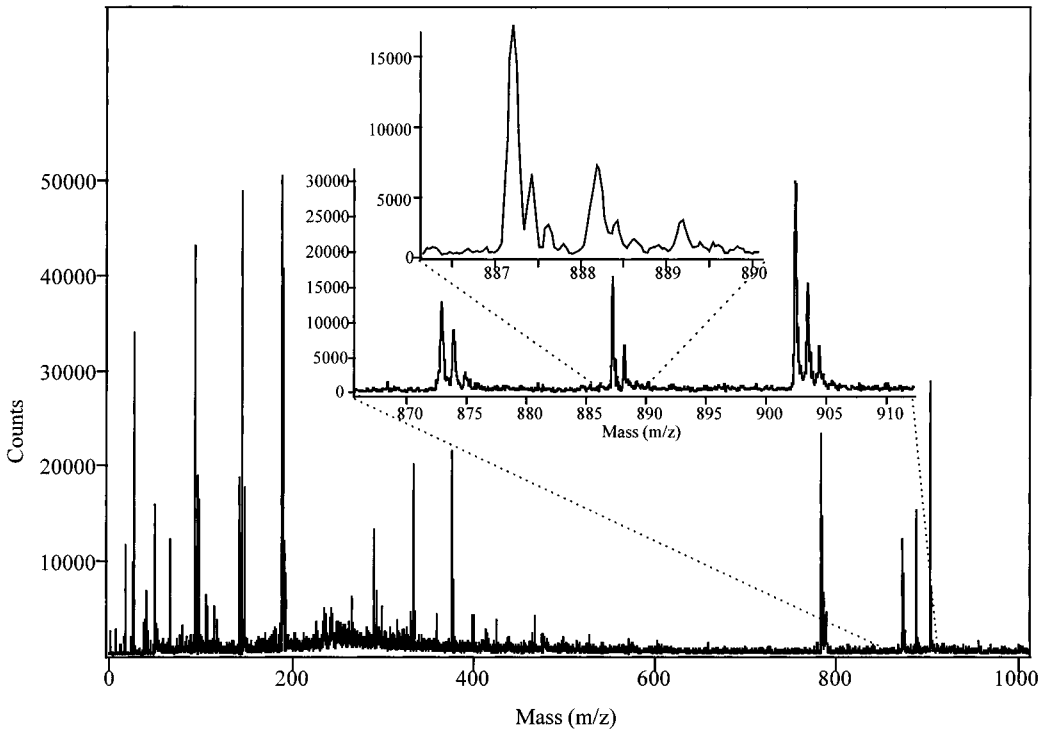

Figure 2. Negative ion MALDI mass spectrum of R-FADH acquired in the delayed extraction reflectron mode (average of 50 laser shots). The ion signal below 400 *m/z* is due to the MALDI matrix α-cyano-4-hydroxycinnamic acid. The first inset shows an expansion of the spectrum in the region of the $[M - H]^-$ ions of R-FADH at *m/z* 887, the internal calibrant des-Arg[1]-bradykinin at *m/z* 902, and the metastable fragment ion (loss of 30) of des-Arg[1]-bradykinin at *m/z* 873. The peak at *m/z* 784 corresponds to two over lapping peaks, both a prompt and metastable fragment ion of R-FADH resulting from the loss of the R group. The second inset further expands the $[M - H]^-$ ion region of R-FADH.

Table 2. Mass Measurement Accuracy of MALDI-DE/rTOF Using Internal Calibration

spectrum no.	R-FADH (*m/z*)
1	887.2043
2	887.2129
3	887.2205
4	887.2143
5	887.2114
6	887.1972
7	887.2166
8	887.2188
9	887.2200
10	887.2220
av obsd	887.2138
std dev (Da)	0.0075
calcd	887.21264
m/z error (Da)	0.0012
m/z error (ppm)	1.4

Table 3. Possible Elemental Compositions for *m/z* 102.0572 ± 0.0068 Da

	C	H	O	N	P	ppm	Δmmu	calcd
*a	0	4	0	7	0	−43	−4.4	102.052 82
a	1	14	0	1	2	29	3.0	102.060 15
b	5	11	0	0	1	26	2.6	102.059 84
b	2	6	1	4	0	−30	−3.0	102.054 16
	4	8	2	1	0	−17	−1.7	102.055 50
b	0	11	2	2	1	−14	−1.4	102.055 81

[a] Eliminated on the basis of the C/H ratio. [b] Eliminated on the basis of the nitrogen rule.

basis of unreasonable C/H ratios or the nitrogen rule.[20] Thus, only a single possibility remains ($C_4H_8NO_2$). The calculated mass of $C_4H_8NO_2$ differs from the measured (via. mass difference) value by only 0.0017 mass unit.

We previously showed that mass measurement accuracy of better than 5 ppm can be obtained for peptides in the mass range of 1−4 kDa using delayed extraction MALDI-RTOF.[15−16] From the data contained herein, it is evident that compounds other than peptides can be analyzed by DE-MALDI/RTOF with high mass measurement accuracy. For example, the average observed *m/z* value for the FAD $[M - H]^-$ ion is 0.0007 mass unit higher than the calculated *m/z*, resulting in a mass error of only 0.9 ppm. This measurement is remarkable considering that this level of accuracy is obtained by applying an external mass calibration.

The mass measurement accuracy often required for new compound identification is 5 ppm; however, in order to unambiguously assign an elemental composition to *m/z* 887.212 64, a mass measurement accuracy of 0.001 ppm is needed. Accuracy of 0.001 ppm is unachievable with current mass spectrometers, but such high accuracy is not necessary to identify the adduct R of R-FADH. After eliminating possibilities from the composition search of *m/z* 102.0572 (Table 3) on the basis of the nitrogen rule or unreasonable C/H ratios, the sole remaining possibility, $C_4H_8NO_2$, corresponds to nitrobutane bound to the N(5) position of the isoalloxazine ring of FAD (Figure 1b), a complete description of the addition of nitrobutane to FAD is explained in detail by Gadda et al.[18]

Mass spectra of R-FADH were acquired with internal calibration (Table 2) because internally calibrated mass spectra are inherently more accurate than externally calibrated spectra. One of the drawbacks of internal calibration is that in some cases the

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calibrant suppresses ionization of the analyte.[2] Therefore, it is necessary to search a range of calibrants to find one that is compatible with the analyte. This procedure is not a problem when an abundant supply of the analyte is available. However, if only a limited amount of analyte is available, it might not be possible to search through different combinations and concentrations of internal calibrants and matrixes to find the combination needed for accurate mass assignment. In the event of a limited supply of sample, the sensitivity of HRMS becomes an issue. Typical FAB HRMS analysis requires nanomoles of material, whereas MALDI-DE/RTOF HRMS analysis requires femtomoles/picomoles of analyte. In this study, 1 pmol of FAD and R-FADH was applied to the probe for the externally calibrated spectra, and 2 pmol of R-FADH was applied for the internally calibrated spectra. Internal calibration yields a mass of 887.2138 (1.4 ppm) for the $[M - H]^-$ ion of R-FADH as compared to 887.2143 (1.9 ppm) obtained by external calibration. By comparing the values obtained by internal and external calibration, it is evident that external calibration is sufficient to determine elemental composition in this case, but until that comparison is made it is impossible to know a priori the accuracy of an external calibration. One means of evaluating an external calibration is by analyzing a known compound similar to the unknown using external calibration. Our analysis of FAD using external calibration yields a mass of 784.1500, a mass error of 0.0007 Da (0.9 ppm). From the accuracy of the mass measurement of FAD, we are confident that external calibration provides the needed mass measurement accuracy in this study.

The utility of accurate mass MALDI-DE/RTOF is shown in the determination of an unknown substituent of a naturally occurring substituted FAD of nitroalkane oxidase isolated from *F. oxysporum*. Accurate mass measurements were acquired with external calibration, and the mass of the unknown substituent was determined from the difference in the observed *m/z* values of FAD and R-FADH. An elemental composition search on the mass of the substituent, after the removal of nitrogen rule violators, yields only one possibility ($C_4H_8NO_2$) corresponding to nitrobutane.

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