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Iterative Accumulation Multiplexing Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (IAM-FTICR-MS)

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Abstract

A multiplexed tandem mass spectrometry (MS/MS) technique known as iterative accumulation multiplexing (IAM) has been implemented on a hybrid quadrupole Fourier transform ion cyclotron resonance mass spectrometer (Q-FTICR-MS). The IAM experiment resulted in obtaining MS/MS spectra for six analytes in two MS/MS experiments while characteristic resolving power and mass measurement accuracies were maintained. Parent-product ion correlations were graphically represented in a “ratiogram” where each product ion is encoded with a ratio unique to the parent ion from which it was formed. This is the first example of multiplexed MS on a FTICR instrument where the ions are encoded externally to the ICR cell. By performing the encoding external to the ICR cell, one set of ions can be encoded while the previous set of ions is being analyzed in the cell, maximizing the use of the continuous ion current emanating from the electrospray ionization source.

Introduction

The use of mass spectrometry as a technique for analyte identification is significantly aided by the ability to perform tandem mass spectrometry (MS/MS) on each analyte of interest. Conventionally, MS/MS is done on one analyte at a time. A variant of MS/MS, referred to as multiplexed tandem mass spectrometry, increases the number of species that undergo MS/MS in a set amount of time. The information generated by carrying out multiplexed MS/MS allows several species to be identified in parallel and signal-to-noise ratios to be improved due to the multiplex advantage. Analyzing multiple parent ions simultaneously is possible by maintaining parent-product ion correlations through an encoding process. 1⁻12

Different encoding schemes have been utilized in multiplexed MS/MS. Several researchers have used Fourier transform ion cyclotron resonance (FTICR) mass analyzers to manipulate parent ions. A variable delay between excitation pulse events to encode a parent ion participating in an ion-molecule reaction has been used to determine a pathway for mass transfer.2 Using the Hadamard transform technique multiplexed MS/MS has been demonstrated on an eleven component mixture as well as to determine all potential dissociation pathways of three different parent ions concurrently.3 A stored waveform ion modulation (SWIM) scheme has been used to analyze up to five ion-molecule proton-transfer reactions simultaneously.4 In each of these examples, the parent ion manipulation results in an encoded signal response that is imparted onto the generated product ions. The product ions can then be correlated (i.e. decoded) to their respective parent ions because

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related ions have undergone the same signal modulation (i.e. encoding process). Other multiplexed approaches have utilized known ion-related properties (e.g. dissociation rate constants or accurate product ion masses) to help decode the convoluted parent-product ion relationships observed when multiple parent ions are encoded and analyzed simultaneously.[5, 6] It has also been demonstrated that parent-product ion correlations can be maintained by encoding the relative amount of each parent ion of interest on quadrupole ion trap (QIT) instruments.[7–10] Two groups have shown that multiplexing based on the accurate mass capabilities of FT-ICR is possible without encoding as long as the proteins are in a database.[13–15]

Iterative accumulation multiplexing (IAM) is a multiplexing approach that encodes the relative amount of ions by modulating individual ion accumulation times.[7, 8] An IAM experiment makes it possible to perform MS/MS on multiple analytes in only two MS/MS experiments. The successful implementation of the IAM experiment requires a mass spectrometer where there is a known relationship between signal response and ion accumulation time. Hybrid quadrupole Fourier transform ion cyclotron resonance mass spectrometers (Q-FTICR-MS) allow ions to be accumulated externally to the ICR cell in a user-defined manner.[16] By accumulating different ions for unique amounts of time the observed abundances of each ion can be modulated thereby encoding the ions of interest. By encoding several ions prior to FT analysis, the multi-channel detection inherent in FTICR-MS allows one transient acquisition to simultaneously provide information on all encoded analytes independently due to the superposition principle. Thus, a reduction in the number of analyses, which is proportional to the number of parent ions being analyzed, is obtained. Because the time to acquire a spectrum in an FT-ICR instrument is relatively long, reducing the number of FT-ICR data acquisitions can substantially decrease the experiment time.[17]

Experimental

Samples

The peptides angiotensin I (DRVYIHPFHL) bradykinin (RPPGFSPFR), granuliberin R (FGFLPIYRRPAS), neurotensin (pELYENKPRRPYIL), and HPLC-grade methanol were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). The peptides substance P (RPKPQQFFGLM; free acid), α-mating factor (WHWLQLKPGQPMY), and cardiodilatin (NPMYNAVSNADLMDFK; 1–16, human) were purchased from the American Peptide Company (Sunnyvale, CA). Water (HPLC grade) and acetic acid (A.C.S. certified) were purchased from Fisher Scientific (Fair Lawn, NJ). All peptides were used without further purification and diluted in 50:50 v% methanol/water resulting in each mixture component being present at a concentration of ~ 5 μM. Acetic acid (1% by volume) was added to the final sample mixture to aid in the electrospray process.

Instrumentation

The experiments were performed on an apex-Qe (Qh-FTICR) mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with a 12-Tesla actively shielded magnet and an Apollo II (Bruker Daltonics, Billerica, MA) electrospray ionization source. The Qh interface is comprised of the mass selective quadrupole (Extrel, Pittsburgh, PA), the accumulation collision cell/hexapole, and their corresponding electronics. The standard apex-Qe electronics require an ICR cell detection event to occur before a different mass-to-charge ratio can be mass selectively passed through the quadrupole. To circumvent this limitation and allow several ions of different mass-to-charge to pass through the quadrupole before they are injected into the ICR cell and detected, modified electronics and software (LabVIEW 7.1, National Instruments, Austin, TX) were used (See Figure S-1 of Supporting Information). The modified electronics are used to control the mode of quadrupole operation

(mass selective or rf only mode), the rf and dc voltage applied to the quadrupole rods to select which analyte to isolate, the collision voltage (relative voltage difference between the dc pole biases applied to the source and collision hexapoles), and the collision hexapole extract bias voltage (dc pole bias applied to the collision hexapole rods when ions are transferred from the collision cell to the ICR cell).

Communication between the main instrument electronics and the modified Qh control electronics and software is accomplished through the use of a trigger output (Collision Cell Pole Bias) from the apex-Qe console. The transition of this trigger to a LO state signals the start of the accumulation event in the collision cell. At this point the custom software is activated and initiates the voltage sequence necessary to execute the IAM experiment. The control program runs on a PXI, Windows XP based computer (PXI8184, National Instruments) housed in a PXI chassis (PXI1002, National Instruments). The PXI chassis also houses an analog output card (PXI6704, National Instruments) that provides the low voltage control signals to the standard Qh electronics and custom power supplies for the collision cell bias voltage. Once the IAM control program is initiated a stepwise procedure commences. At each step the mass selective quadrupole rf and dc voltages are set to pass a given mass-to-charge value ("*m/z*-equivalent rf/dc control voltage"). Additionally the collision voltage for that mass-to-charge is set ("Collision Cell Bias Voltage") so that the desired dissociation is achieved. A unique collision cell bias voltage can be set for each ion (over the range of 0 to −150 V, for positive ions) so different collision induced dissociation (CID) conditions can be used for every analyte if so desired. These voltages are set for the desired IAM accumulation time and the process is repeated for every parent ion of interest. The user enters the IAM *m/z* value of interest, its collision energy, and accumulation time into the custom LabVIEW control software prior to starting the instrumental scan.

IAM Procedure

Protonated molecules are generated by ESI and sequentially selected by the quadrupole and accumulated in the collision hexapole as dictated by the LabVIEW control software discussed above. CID is accomplished by transferring ions into the collision hexapole through a user-specified voltage drop established between the source and collision hexapoles. Unless otherwise noted, the CID voltage drops used for bradykinin, substance P, granuliberin R, neurotensin, α-mating factor, and cardiodilatin were 18.00 V, 22.00 V, 28.00 V, 33.00 V, 26.00 V, and 24.00 V, respectively. All the ions in the hexapole are then injected into the ICR cell for detection (50 scans were averaged for all spectra).

The key aspect to IAM is that two spectra are collected, each at different relative accumulation times for the parent ions of interest. A "ratiogram" is then generated by dividing ion abundances from the two acquired spectra. As an example, the following is a description of how an IAM experiment for a three-component mixture comprised of ions A, B, and C would be implemented. The first of two spectra (Spectrum I) is acquired with the relative A:B:C accumulation times of 1:1:2. (The absolute accumulation times, milliseconds, tens of milliseconds or hundreds of milliseconds, are not important unless the total time exceeds the time necessary to acquire the mass spectrum in the FT-ICR). While the first FT-ICR spectrum is being acquired ions for the second spectrum (Spectrum II) are being accumulated in the hexapole with different relative accumulation times (e.g. 1:2:1). The second FT-ICR spectrum is then acquired. Assuming a constant flow of ions from the electrospray source and by keeping all instrumental parameters the same except the relative accumulation times, changes in signal response should be due only to changes in accumulation time. Thus by dividing the spectral abundances of Spectrum I by those of Spectrum II, a ratiogram is generated where each species is seen to have a unique ratio (A:B:C = 1:0.5:2). The incorporation of CID (in the collision cell) into the IAM experiment results in product ions acquiring the same ratio that was imparted to their respective parent

ion during the encoding process. Consequently, each peak in the ratiogram can be assigned to its parent ion by virtue of the encoding.

Results and Discussion

The successful implementation of an IAM experiment is predicated on there being a known relationship between ion accumulation time and signal response. Control experiments were performed on the Q-FTICR-MS instrument to determine the linearity between ion accumulation time in the collision hexapole and ion abundance detected by the FTICR cell. In the control experiment, protonated molecules of angiotensin I ($[M + 3H]^{3+}$), bradykinin ($[M + 2H]^{2+}$) and substance P ($[M + 2H]^{2+}$) were passed sequentially through the mass-selective quadrupole for equal amounts of time. Once all three sets of ions had been accumulated in the collision hexapole, the total ensemble of ions was sent to the ICR cell for mass analysis. The results illustrated that under this specific charge density there is a linear signal response ($R^2 = 0.9949$) for a total accumulation time of less than ~600 ms (i.e., the sum of three individual ion accumulation times of 200 ms). At a total accumulation time longer than 600 ms the plot of abundance versus accumulation time departs from linearity indicating that the space charge limit of the collision hexapole had been reached. The space charge limit represents the largest amount of charge that can be stored within a given trapping volume. Because two spectral acquisitions are required to generate the ratiogram for an IAM experiment, it is necessary to work with accumulation times that correspond to the linear portion of the signal response curve. Thus, the total ion accumulation time for peptide IAM experiments was kept ≤ 600 ms.

IAM experiments were performed on the Q-FTICR-MS instrument using a six-peptide mixture. The encoding parameters and resulting peak abundances for the six analytes are listed in Table 1. The amount of time each parent ion was accumulated in the collision hexapole before being detected to generate Spectrum I is listed in the second row of Table 1. Also in the second row, the monoisotopic peak abundances are listed for each parent ion as determined by Bruker Daltonic's SNAP2 algorithm within the Bruker Data Analysis software suite. Row three contains the accumulation time and resulting monoisotopic peak abundances for Spectrum II. The theoretical ratios expected from this encoding scheme are listed in row four for each peptide, calculated by dividing the Spectrum I accumulation times by the Spectrum II accumulation times. The observed ratios in row five were calculated by dividing the abundances of the respective species in Spectrum I by their corresponding abundances in Spectrum II. For the peptides considered, it was empirically determined that having a constant total accumulation time allowed for better agreement between theoretical and observed ratios in most instances. The reason for this better performance is still under investigation. However, preliminary results suggest that the behavior may be related to the ability to duplicate possible space charge or ion loss conditions, or both, in Spectrum I and II by keeping the total accumulation time constant. In a separate set of experiments to quantify the instrumental scan-to-scan reproducibility, five consecutive scans of an equal-molar mixture of bradykinin, substance P, and granuliberin R were acquired. The results showed that the largest relative standard deviation for scan-to-scan peak abundance and observed parent ion monoisotopic peak centroid value were 4.56% and 4.64×10^{-6}%, respectively. This reproducibility adds confidence to the encoding procedure because two consecutive scans are required to generate a ratiogram.

Encoding the ions using the accumulation times listed in Table 1 and performing CID (IAM-CID) resulted in the theoretical ratios for the parent ions of interest being imparted to their respective product ions. As shown in Figure 1A conducting CID on six parent ions simultaneously produces complex spectra. The application of the IAM procedure is illustrated in Figure 1B where the monoisotopic and ^{13}C peaks for selected product ions

from granuliberin R, cardiodilatin, and substance P are overlaid. The theoretical ratio for each parent ion, and consequently each product ion, is listed in parentheses, preceded by the observed ratio. As can be seen, the encoding scheme is effectively transferred to the product ions allowing them to be unambiguously assigned to their respective parent ion. There are two important features in the spectra shown in Figure 1B. First, the substance P a_9-NH_3 and the cardiodilatin y_9 product ions could be identified as coming from different parent ions based solely on their observed ratios. Second, the a_9-NH_3 substance P peak was acquired with a mass resolving power of 26,947 which is sufficient to allow it to be resolved from the ^{13}C peak of y_9 cardiodilatin. The high resolving power offered by FT detection allows product ion information to be retained during the IAM-FTICR-MS process that would have been lost when using QIT mass spectrometers to perform IAM experiments.[7, 8] No effort was made at maximizing, or even optimizing, the resolving power of this instrument while performing the experiment shown in Figure 1. This easily achievable resolving power was sufficient to get analytically useful results.

To aid in simplifying data interpretation, the product ions generated from the two CID spectra (Spectrum I and II) can be represented in a ratiogram, as shown in Figure 2. Monoisotopic peak lists were generated using Bruker Daltonic's SNAP2 algorithm. The monoisotopic peak lists for Spectra I and II were divided to create the ratiograms. A mass-to-charge tolerance of 5 ppm was used to ensure that the same product ion was used in Spectra I and II to calculate a given ratio. The product ions associated with each ratio were annotated by comparing observed mass-to-charge values to those generated *in silica* by the MS-Product functionality within Protein Prospector (Protein Prospector 4.0.8, University of California). The average mass measurement accuracy for the product ions in Figure 2 was 0.47 ppm, indicating that the high mass measurement accuracy typical of this instrument was maintained during the IAM experiment. The ratios are grouped around their respective theoretical ratios allowing the product ions to be discerned from one another. Some of the observed ratios in Figure 2 are slightly higher than their theoretical values. Experiments are currently being conducted to gain a better understanding of ion accumulation in the collision hexapole, and ion transfer to and ion storage in the ICR cell. Better agreement between theoretical and observed ratios may be attainable through the use of multiple ICR cell fills with a shorter collision hexapole accumulation event to decrease the affect of space charging within the hexapole. Such effects have been shown to introduce a non-monotonic dependence of ICR signal on external ion accumulation time.[18] However, the absolute value of the ratio is not important, only the ability to differentiate between ratios.

Also in Figure 2, there are instances where a product ion was observed at a mass-to-charge ratio that could represent a product ion from more than one of the potential parent ions. For example, the product ion at *m/z* 226.1186 could represent the isomers PQ, from substance P, QP from α-mating factor, or NK-NH_3 from neurotensin. Because the product ions in question are isomers the mass measurement accuracy and resolving power achievable from the FT detection does not help in determining which parent ion (e.g., substance P, α-mating factor, or neurotensin) produced *m/z* 226.1186. Thus, techniques that do not encode the ions could not determine the appropriate parent ion.[13–15] However, due to the encoding scheme used in the IAM experiment the parent-product ion relationship for *m/z* 226.1186 can be determined assuming only one parent ion fragments to that particular product ion. If more than one parent ion fragments to the same isomeric product ions then the ratio should be a linear combination of the ratios of the parent ion encoding scheme, which may or may not give ambiguous results. In the present example, the product ion at *m/z* 226.1186 can be confidently annotated as the PQ internal fragment from substance P. A similar situation was observed for four potential isomeric product ions at *m/z* 254.1611 and also for two isomeric product ions at *m/z* 1171.6735. Control experiments were performed where each parent ion was individually isolated and dissociated by CID at the same conditions used for the IAM

experiment. The product ions generated from the control experiments corroborate the identifications made via the IAM experiment. The correct annotation of these isomeric product ions verifies the utility of the encoding process to accurately assign parent-product ion correlations for isomeric product ions that are only formed from one parent ion.

In Figure 2, at least one product ion for each parent peptide was observed, however there are a limited number of product ions for certain parent ions (e.g. granuliberin R and α-mating factor). To determine if the IAM process is affecting the parent ion dissociation, control experiments were performed where each peptide was dissociated individually and the extent of their dissociation was compared to the extent each peptide was dissociated during IAM. The extent of dissociation can be represented by MS/MS efficiency, defined here as (Σ(product ion abundances) / initial parent ion abundance). The results of this comparison are given in Table 2. For the control experiments, each peptide was individually isolated from the six-peptide mixture, accumulated for 100 ms (chosen because it is the average of Spectrum I or Spectrum II accumulation times), and dissociated using the same CID voltage utilized during the IAM experiment (as listed in the Experimental section). The MS/MS efficiencies listed in Table 2 for the control experiments are the average (± one standard deviation) of three trials. For the IAM efficiencies, the IAM experiment described by Table 1 was performed with and without CID in duplicate. Thus for each IAM experiment, Spectrum I was acquired without CID and with CID as was Spectrum II so MS/MS efficiencies could be calculated for each parent ion under both Spectrum I and II conditions. Therefore the efficiencies listed in Table 2 for each parent ion is the average of four MS/MS efficiency values.

For a given set of IAM experiments only product ions in the CID ratiogram attributable to a given parent ion were considered in calculating the MS/MS efficiencies. The reason for only using product ions found in the CID ratiogram is shown in Figure 3. The CID spectrum of $[M+2H]^{2+}$ cardiodilatin acquired conventionally is shown in Figure 3A. Figures 3B and 3C are the reconstructed CID spectra of $[M+2H]^{2+}$ cardiodilatin under Spectrum I and II conditions from Table 1, respectively. The annotated peaks in Figure 3 correspond to those for cardiodilatin in Figure 2. Despite there being more product ions present in Figure 3B, for the decoding step of the IAM process (i.e., Spectrum I / Spectrum II) to provide meaningful results, only the abundances of those ions present in both Spectrum I and II can be considered. Therefore, even though the reconstructed CID results for Spectrum I contain more product ions than Spectrum II, the number of product ions that can be included in the ratiogram is limited to those present in the spectrum with the fewest number of product ions, i.e. Spectrum II. The result of this limitation is that IAM efficiencies would be expected to be lower than the control experiments, because fewer product ion abundances would be considered in the MS/MS efficiency calculation (e.g., there are 13 product ions in Figure 3C and 33 product ions in Figure 3A). While the results from Table 2 support this idea, it should be emphasized that the IAM process itself is not the cause for the reduced MS/MS efficiencies. Rather, the limitation is the relatively short parent ion accumulation times (e.g., 50 ms) used for the peptide IAM experiments presented in Figures 2 and 3 and Table 2. By reducing the accumulation time the absolute number of product ions formed and stored in the collision hexapole is decreased, resulting in a smaller signal response from the ICR cell detection. Consequently, the resulting ICR signal may not produce a signal-to-noise ratio sufficient enough for those ions to be detected. To avoid such a situation, the IAM encoding scheme could be changed to incorporate longer accumulation times. As long as the total encoding time is shorter than the time to acquire the transient image current, there is no loss of duty cycle because while one set of ions is being detected, the next set is being prepared for injection into the ICR cell.

One potential concern is variation in the fragmentation of a given parent ion based on how long it is held in the hexapole during the accumulation. There are two possible scenarios that could lead to such an outcome. One is the increased number of collisions experienced by the parent ions accumulated in the hexapole at the beginning of the accumulation. Another scenario is multipole storage assisted dissociation (MSAD).19 MSAD occurs when there are a large number of ions stored in the hexapole. In Figure 3 it can be seen that conventional MS/MS spectrum is very similar to the reconstructed MS/MS spectra, indicating neither of the above possibilities are occurring. While certainly the ions stored longer in the hexapole will undergo more collisions, after a certain point these ions have been collisionally cooled (and have lost kinetic energy in the partitioning of the kinetic energy between the fragments when the ion dissociates) and further collisions have little impact on the ion internal energy. One has to be aware of the potential of MSAD, but that would likely involve conditions beyond where there is a linear response as a function of injection time as discussed above. To further address this possibility experiments were done in which the order of accumulation was changed and no significant changes in the MS/MS spectra or ratiograms were observed.

A second IAM experiment was performed using the same analytes listed in Table I, but with different relative accumulation times. The altered encoding scheme resulted in the doubly protonated parent ions of bradykinin, substance P, granuliberin R, neurotensin, α-mating factor, and cardiodilatin having requested ratios of 3.5, 2.0, 1.2, 0.8, 0.5, and 0.3, respectively. The ratiogram for this experiment is shown in Figure S-2 of the Supporting Information. These ratios are approximately the inverse of those listed in Table 1. For this second IAM experiment the requested ratio for α-mating factor was kept the same as the previous experiment (i.e., 0.5) but the Spectrum I accumulation time was increased to 75 ms. By increasing the accumulation time three α-mating factor product ions were observed in the ratiogram, compared to only one when a Spectrum I accumulation time of 50 ms was used in the first IAM experiment. Also demonstrated in the ratiogram of Figure S-2 is the independence of requested ratio on mass-to-charge value of the encoded parent ions. For example, the encoding of cardiodilatin in the first and second IAM experiments was 3.0 and 0.3, respectively. As the ratiograms in Figure 2 and Figure S-2 indicate, the product ions for cardiodilatin are accurately encoded for both conditions.

Conclusions

Iterative accumulation multiplexing (IAM) has been successfully implemented external to the ICR cell on a hybrid quadrupole-FTICR-MS instrument. The IAM experiment allowed MS/MS of six analytes to be performed in two MS/MS experiments without sacrificing ICR performance (i.e. mass measurement accuracy or mass resolving power). Product ion information generated by IAM is provided by ratiograms that are comprised of species recorded with high mass measurement accuracy. Due to the encoding process, the abundances of the peaks in the ratiogram allow the product ions to be unambiguously assigned to their corresponding parent ion. We believe more than six ions can be multiplexed. The limitation is the space charge capacity of the accumulation hexapole (or some other accumulation device such as a linear ion trap in a hybrid instrument). The high resolution capability of the ICR significantly reduces uncertainty in the determination of the ratiograms, allowing more ions to be multiplexed in this instrument than in a quadrupole ion trap.

In this proof of principle direct infusions was used. To increase the utility of IAM it would be advantageous to implement IAM during LC-MS/MS experiments. It may be possible to implement IAM in this scenario without using an encoding algorithm, but just using the different elution profiles of the analytes as the means of encoding. For example, if Spectra I

and II are acquired at different locations on the chromatographic peak (i.e., different ion abundances) and a constant accumulation time is used for all ions, one would not a a priori know the encoding ratio, but as long as it is different that does not matter.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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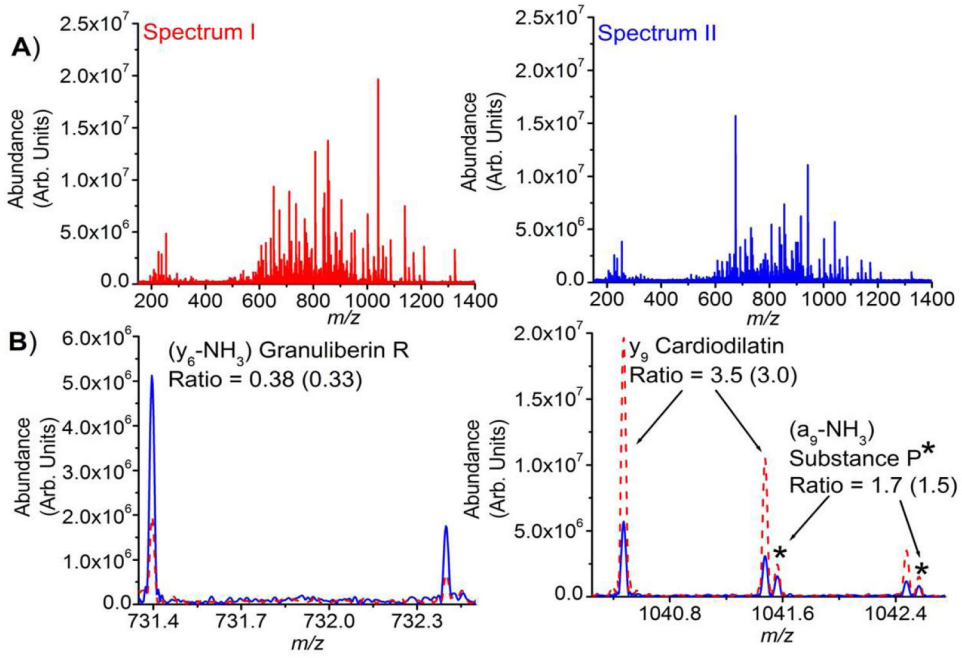

Figure 1.

(A) CID spectra that resulted from the encoding scheme in Table 1; (B) Monoisotopic and ^{13}C peaks for selected product ions overlaid from Spectrum I (red, dashed) and Spectrum II (blue, solid) around cardiodilatin, granuliberin R, and substance P. The asterisks in the cardiodilatin spectrum denote the a_9-NH_3 peak of substance P (see text).

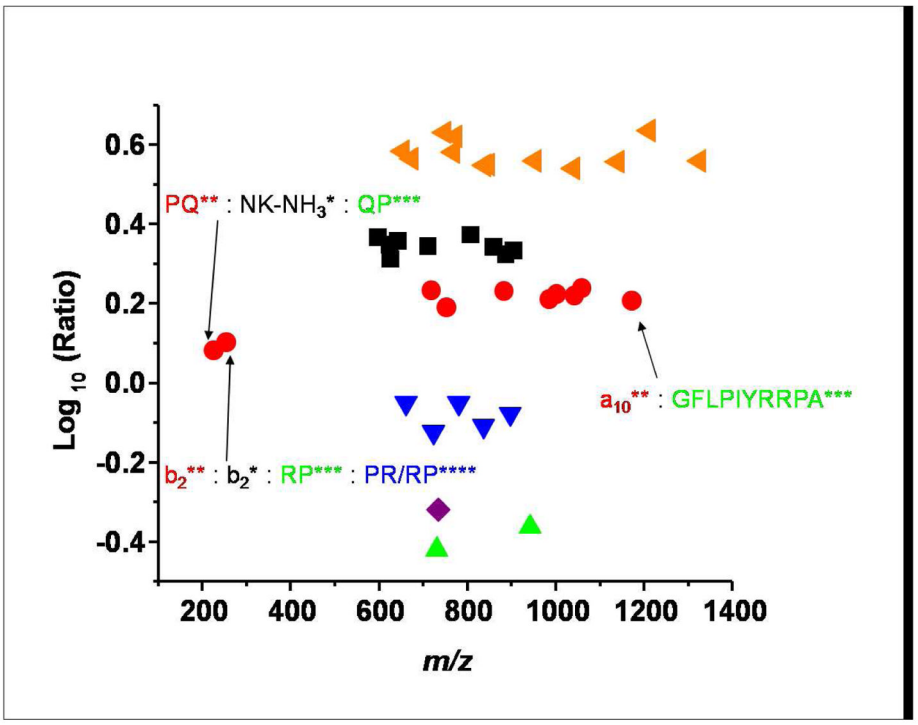

Figure 2.

Ratiogram from the IAM-CID experiment using parent ions and encoding scheme outlined in Table 1. The product ions labeled with asterisks are isomers of the labeled mass-to-charge value. The correct annotation for an isomeric product ion in the ratiogram is the first one listed for a given mass-to-charge value (see text). Each asterisk (or multiple asterisk) corresponds to a specific parent ion: bradykinin (■)*; substance P ●**; granuliberin R ▲***; neurotensin ▼****; α-mating factor (♦); cardiodilatin (◀).

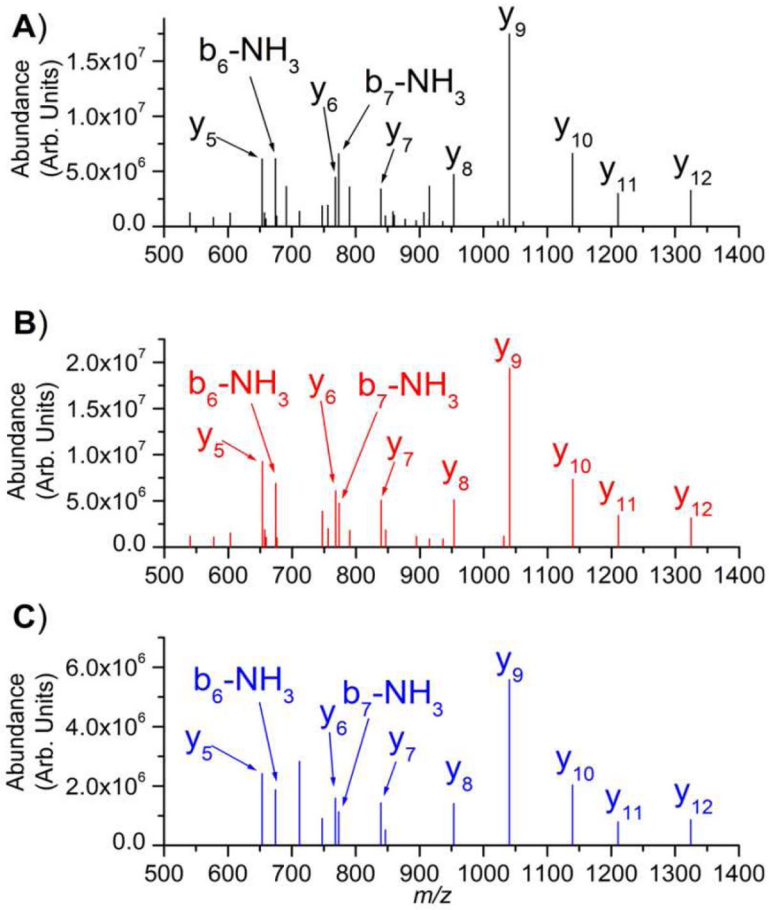

Figure 3.

CID spectra of $[M+2H]^{2+}$ cardiodilatin: (A) CID spectrum after individually isolating and accumulating the parent ion; (B) Product ion spectrum reconstructed from an IAM experiment performed under the Spectrum I conditions given in Table 1 for cardiodilatin; (C) Product ion spectrum reconstructed from an IAM experiment performed under the Spectrum II conditions given in Table 1 for cardiodilatin.

Table 1

Encoding scheme used for peptide IAM experiments. The theoretical ratios are calculated by dividing the Spectrum I accumulation times by the Spectrum II accumulation times. The observed ratios are determined by dividing the Spectrum I abundances by the Spectrum II abundances.

Peptide	**$[M + 2H]^{2+}$ bradykinin**	**$[M + 2H]^{2+}$ substance P**	**$[M + 2H]^{+2}$ granuliberin R**	**$[M + 2H]^{+2}$ neurotensin**	**$[M + 2H]^{+2}$ α-mating factor**	**$[M + 2H]^{+2}$ cardiodilatin**
m/z	**530.7880**	**674.8634**	**711.9039**	**836.9621**	**842.4268**	**915.4135**
Spectrum I Time, ms	100	150	50	100	50	150
Parent Ion Abundance (Arb. Units)	6.2×10^{7}	5.1×10^{8}	1.0×10^{8}	2.2×10^{8}	7.4×10^{7}	3.0×10^{8}
Spectrum II Time, ms	50	100	150	150	100	50
Parent Ion Abundance (Arb. Units)	2.7×10^{7}	3.0×10^{8}	2.5×10^{8}	2.9×10^{8}	1.2×10^{8}	8.9×10^{7}
Theoretical Ratio	2.0	1.5	0.33	0.67	0.50	3.0
Observed Ratio	2.3	1.7	0.40	0.76	0.62	3.4

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Table 2

MS/MS efficiency comparison between the control and IAM conditions.

Peptide	Control Experiment MS/MS Efficiencies n = 3 (%)†	Spectrum I Time, ms	Spectrum II Time, ms	Requested Ratio	IAM Experiments MS/MS Efficiencies n = 4 (%)‡
bradykinin	46.79 ± 1.70	100	50	2.0	30.99 ± 2.87
substance P	18.80 ± 1.10	150	100	1.5	11.64 ± 0.67
granuliberin R	11.97 ± 0.36	50	150	0.33	4.14 ± 0.70
neurotensin	10.34 ± 0.91	100	150	0.67	9.29 ± 2.53
α-mating factor	18.85 ± 2.61	50	100	0.50	13.07 ± 1.80
cardiodilatin	67.93 ± 4.05	150	50	3.0	21.25 ± 2.61

†The control experiment (see text) was performed on each peptide in triplicate.

‡Four MS/MS efficiency values (see text) for each parent ion were averaged to determine the IAM MS/MS efficiency.