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Dynamic Collision-Induced Dissociation of Peptides in a Quadrupole Ion Trap Mass Spectrometer

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The fragmentation of natural peptides using dynamic collision-induced dissociation (DCID), a novel fragmentation method for quadrupole ion traps, is demonstrated. Using leucine enkephalin as a diagnostic molecule, the fragmentation efficiencies and energetics of DCID are compared with other methods of collisional activation in ion traps such as conventional on-resonance excitation and high-amplitude short-time excitation (HASTE). A typical fragmentation efficiency of ~20% is achieved for DCID, which is significantly lower than conventional CID (maximum near 80%). Tandem mass spectra of two other peptides, substance P and oxidized insulin α-chain, demonstrate that product ion spectra for DCID are comparable to conventional or HASTE CID. Because DCID achieves fragmentation during the standard mass acquisition scan, no extra time is necessary for on-resonance excitation or product ion collection, so analysis times are reduced by a minimum of 10−15% depending on the scanning conditions. DCID therefore offers more tandem mass spectra per second than conventional methods of collisional activation, which could be highly advantageous for bottom-up proteomics separations.

Quadrupole ion traps (QITs) were first introduced on the commercial market as mass spectrometers in the mid 1980s following the work of Stafford and co-workers.[1] This work was soon followed by the demonstration by Louris et al.[2] that tandem mass spectrometry could be performed in real time by exciting the ions with the help of an auxiliary rf frequency applied to the end-cap electrodes. Louris et al. showed that when the excitation frequency matched the secular frequency of ions of a given mass-to-charge ratio, collision-induced dissociation (CID) could be achieved. The use of ion traps has been growing ever since, as demonstrated by the number of commercial instruments available on the market and the number of publications and applications in the chemical and biomedical fields. QITs continue to be refined and improved as demonstrated by the recent development of digital ion traps,[3−5] miniature traps,[6,7] linear ion traps,[8,9] and ion−ion reaction technologies.[10−12] The major limitations of QITs are the relatively slow data acquisition rates (1−5 Hz) and the low-mass cutoff value at which CID is performed, which limits the ability to recapture low-mass product ions.

In developing methods to improve the capabilities of QITs, it is important to understand the various waveforms that are typically applied to QITs to either fragment or eject ions. Resonance ejection or axial modulation is achieved by ejecting ions from the trap before they reach the limits of the Mathieu stability diagram ($q_z = 0.908$, when $a_z = 0$) during the mass instability scan. Resonance ejection is achieved by applying a supplementary ac voltage on one or both end-caps at a frequency equal to the secular frequency of ions of a given Mathieu stability parameter (q_z). This method was first described by Fulford and co-workers[13] and was demonstrated to have a beneficial effect on mass resolution when combined with mass instability scanning.[14,15] Resonance ejection permitted the extension of the mass scanning range available in ion traps,[16,17] which tremendously increased the versatility of QITs. However, resonance ejection also has deleterious effects on the

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mass spectrum and can cause mass shifts,[18,19] peak fronting,[20] and ghost peaks.[21] These problematic phenomena are usually observed when space-charge broadens the distribution of secular frequencies of a packet of ions of a given m/z or when fragile ions dissociate during the resonance ejection process. These issues lead to a loss in mass resolution or misleading spectra.

To perform tandem mass spectrometry, ions are typically excited, fragmented, and recaptured during a separate scan function prior to the mass analysis scan. The most common fragmentation method is on-resonance CID, which—like resonance ejection—relies on applying a supplementary ac voltage to the end-cap electrodes. To achieve CID, excitation amplitudes are selected that enable the internal energy of the ions to be increased via collisions with bath gas, but sufficiently small as to prevent resonance ejection. On-resonance CID has been well studied and is known to be dependent on a number of variables including excitation time, excitation amplitude, and trapping parameters; which influence the efficiency and energetics of the fragmentation.[2,22−24] Each of these parameters—and the presence of higher-order fields inside an ion trap—can slightly shift the secular frequency of ions before or during on-resonance excitation, which makes it difficult to apply the "ideal" on-resonance waveform. To circumvent this problem, other dissociation methods that do not rely on on-resonance excitation, such as boundary-activated dissociation,[25,26] infrared multiphoton dissociation,[27,28] electron-transfer dissociation,[29,30] surface induced dissociation,[31] and red-shifted off-resonance large-amplitude excitation[32] have been proposed. However, none of the methods has yet found widespread use. Recently, three groups have proposed an on-resonance excitation approach that could improve the speed of acquisition, the fragmentation energetics, and the dynamic mass range with which fragment ions are recaptured.[33−35] Typically, on-resonance CID requires tens of milliseconds of excitation (~30 ms), but "fast excitation",[33] pulsed Q dissociation (PQD),[35] and high-amplitude short-time excitation (HASTE)[34] CID excite ions with a higher amplitude but for much shorter periods: Fast CID and PQD utilize ~100-μs pulses of on-resonance excitation to excite ions, whereas HASTE uses pulse widths of 1−2 ms. These methods offer the advantage of imparting more internal energy to the ions while permitting the recapture of fragment ions of low m/z by quickly lowering the primary rf amplitude after excitation—to effectively lower the q_z—before fragmentation occurs. Depending on the scanning parameters, using one of these methods could cut total scan time by as much as 30%. Although these methods offer time savings and wider mass range of collected product ions than conventional CID, on-resonance conditions must still be obtained in order to achieve efficacious and reproducible results.

The reduction of analysis time for tandem mass spectrometry becomes important with the availability of faster and more complex separation techniques that produce peaks lasting less than 1 s. To complement the excitation methods mentioned above, a method of fragmentation during mass acquisition, known as dynamic CID (DCID) was recently described and demonstrated to promote fragmentation on a level at least comparable with the other methods for the analysis of small molecules.[36,37] DCID is performed by applying a supplementary axial modulation frequency to the end-caps during the mass acquisition scan. The resonance ejection frequency remains unmodified to achieve enhanced mass resolution, but an excitation waveform with a smaller frequency and smaller amplitude is applied to match the frequency of ions at a predetermined q_z, typically somewhere between $q_z = 0.25$ and $q_z = 0.45$. As the primary rf amplitude is ramped to perform the mass instability scan, the ions' secular frequency increases. At some point, the secular frequency matches the excitation frequency and the ions gain kinetic energy that can be sufficient to cause fragmentation via collisions with the bath gas. All the fragments that have a mass-to-charge ratio above the low-mass cutoff (LMCO) at the time of formation can be recaptured and mass-selectively ejected at their proper mass-to-charge value when they reach the resonance ejection point. The advantage of this method is that it eliminates the need for a separate excitation period, which means that the entire scan time is shorter. The second advantage is that there is no need to tune the excitation frequency to match exactly the secular frequency of the ions because the secular frequency of the ions is being "scanned" across the excitation frequency as the mass scan is being performed. This method is, in some fashion, inspired from modulated resonant excitation,[38] where the drive frequency is oscillated back and forth over a narrow-amplitude window, to ensure the excitation of all of the precursor ions, even if there is a slight shift in their secular frequencies. The main downside to this approach is that the fragmentation and mass instability scan take place simultaneously, so they cannot be optimized independently.

The need for higher throughput tandem mass spectrometric techniques for the analysis of peptides has been recently documented by Shen and co-workers.[39] The shorter the mass scanning

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function, the greater the number of tandem spectra that can be acquired during the elution of a chromatographic peak, and the greater the chance of obtaining complete proteomic coverage. The present paper demonstrates the use of DCID for the dissociation and study of different natural peptides. The results are compared with results obtained on the same instrument using conventional CID and HASTE fragmentation methods for leucine enkephalin, substance P, and oxidized insulin α-chain.

EXPERIMENTAL SECTION

Experiments were conducted on a Bruker Daltonics (Billerica, MA) EsquireLC mass spectrometer. All analyses were performed using the normal scan rate (13 000 Th/s). Instrument parameters optimized for each peptide included capillary voltage (3500−4000 V), skimmer 1 (30−40 V), trap drive (LMCO) (50−60 Th), drying gas flows (5−7 L/min), and drying gas temperature (200−300 °C) as well as the accumulation time (3−10 ms). Other parameters were kept constant throughout: endplate offset (−500 V), octopole voltage (2.8 V), octopole Δ (2.4 V), octopole rf (150 V_{pp}), skimmer 2 (6 V), lens 1 (−5 V), and lens 2 (−60 V); the polarity of all voltages was inverted for insulin α-chain analysis. The peptides were individually introduced via direct injection at a flow rate of 2 μL/min using a syringe pump (Cole-Parmer 74900-00, Vernon Hills, IL).

The system was used either in its standard operating mode with no modifications for CID and HASTE experiments or with an external waveform being applied to the entrance end-cap (connector J124) to perform DCID. For DCID experiments, the grounding plug on connector J124 was replaced with the output of an arbitrary function generator (Agilent 33250A, Palo Alto, CA) during the mass acquisition scan. A filtering circuit was included in between the function generator and the entrance cap electrode to protect the function generator from the rf potential induced on the end-caps by the ring electrode. The excitation waveform applied was an 81-kHz sine wave, which, for this particular instrument (Ω = 781 kHz) provides resonant excitation at approximately $q_z = 0.29$. Because of the slight amplifying effect of the low-pass filter, the excitation amplitudes actually applied to the entrance end-cap were measured at the J124 terminal using the FFT function on a digital oscilloscope (Tektronix TDS 3032B, Richardson, TX). In the normal operation of the Esquire LC instrument, the supplementary ac waveform used for resonance ejection or excitation is only applied to the exit end-cap (connector J125); no modifications were made to the signals applied to this end-cap. The capillary exit voltage supply (connector J43) on the buffer panel was used as a trigger signal; the signal was reduced to a TTL using a series of resistors. The trigger marks the beginning of the ion accumulation period, and a delay was added to synchronize the applied external excitation waveform on the entrance end-cap with the start of the analytical mass acquisition scan. Figure 1 illustrates the differences between scan functions used for conventional CID and DCID. In these experiments, the duration of the fragmentation period were set at 30 ms for conventional CID and at 2 ms for HASTE.

Leucine enkephalin (YGGFL) and oxidized insulin α-chain (GIVEQCoxCoxASVCoxSLYQLENYCoxN) were obtained from

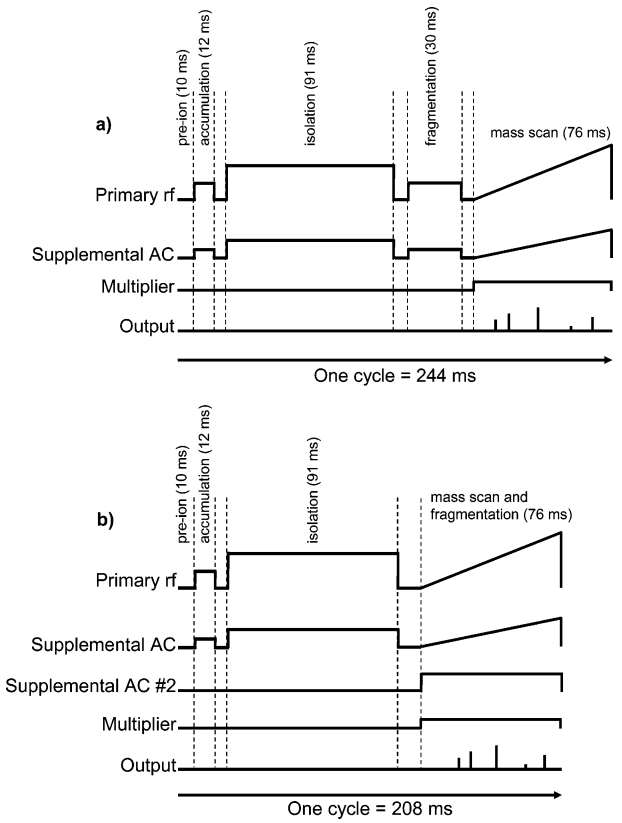

Figure 1. Representation of the typical scan functions used for (a) conventional CID and (b) dynamic CID (DCID). Example for the acquisition of leucine enkephalin, mass range 50−1000, on Bruker EsquireLC.

Sigma-Aldrich (St-Louis, MO), and substance P (RPKPQQFFGLM-NH_2) was purchased from Calbiochem (San Diego, CA). Water was purified using a Milli-Q system (Millipore, Billerica, MA), HPLC grade methanol was purchased from EMD (Gibbstown, NJ), and 99.99% acetic acid was from Sigma-Aldrich. The 100 μM standards for leucine enkephalin and substance P were prepared by dissolving each one in a 50:49:1 mixture of methanol/water/acetic acid. Dilutions using the same solvent mixture were performed to obtain final concentration of ∼10 μM for both peptides. In the case of oxidized insulin α-chain, a standard was prepared in 9:1 methanol/water solvent at a concentration of 100 μM and further diluted to a working concentration of 20 μM.

RESULTS AND DISCUSSION

To compare conventional CID with DCID, a series of experiments were performed using three well-characterized peptides of different molecular weights, charges, and polarities. Oxidized insulin α-chain and substance P were used as multiply charged anion (4−) and cation (2+), respectively, while leucine enkephalin was studied as a singly protonated cation. The two main fragments present in the mass spectra of leucine enkephalin, a_4 and b_4, were used as "indicator" fragments to determine the efficiency and energetics of the fragmentation of different excitation waveforms. The LMCO for both CID and HASTE experiments was determined by ensuring that the applied excitation frequency matched the 81 kHz used for the DCID experiments, representing a stability parameter of $q_z = 0.29$. The actual LMCOs required were therefore 180, 220, and 205 Th for leucine enkephalin, substance P, and

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oxidized insulin α-chain, respectively. As described in a previous study,[36] DCID seems to allow higher energy fragmentation pathways to be attained than conventional CID. This is thought to derive from the faster energy deposition rates in DCID versus conventional CID. Since the ions' secular frequencies are continuously changing with the ramping of the drive rf amplitude, the ions are only in resonance with the excitation frequency for a brief period. With larger excitation amplitudes, the ions gain more internal energy with fewer collisions and more of this energy goes toward fragmentation since there is less chance for energy dissipation through collisional cooling as is the case with longer (30 ms) excitation periods used in conventional CID. These observations are in agreement with the results of Murrell and coworkers[33] that good energetics can be achieved with a short excitation period, but with a loss in CID efficiency.

The results for the CID efficiencies and ratio of a_4/b_4 ions for the fragmentation of leucine enkephalin are presented in Figure 2. The percent CID efficiency is calculated from the sum of the intensities of all the fragment ions as a numerator and the intensity of the isolated precursor ion (556.5 Th) with 0 V excitation amplitude, collected in a prior scan, as the denominator. The average relative standard deviation on the percent CID efficiency for each of the three fragmentation methods was 6% ($n = 9$). Error bars were not plotted because they made the figures difficult to interpret. The largest variations (19%) were observed for the lowest measured CID efficiencies. The most precise CID efficiencies were obtained near the maximum CID efficiencies. For the dissociation energetics, the relative standard deviations averaged just under 15% for HASTE and conventional CID and 17% for DCID for three replicates of the a_4/b_4 ratio. The relative standard deviation spread between 4.4−29.8%, 1.7−40.2%, and 3.8−37.3%, respectively, for DCID, HASTE, and conventional CID. The largest absolute errors were observed for the three highest energetic points obtained with conventional CID (Figure 2c).

The results in Figure 2 show that fragmentation efficiency in DCID is quite constant over the range of excitation amplitudes studied, although the reasons for this behavior are not well understood. Conventional CID achieves the largest internal energies with a_4/b_4 ratios around 4.5 over a narrow range of excitation amplitudes, but this occurs at CID efficiencies below 30%, which is outside of the more efficient normal working range of 70−80%. Conversely, DCID can achieve highly energetic fragmentation, a_4/b_4 ratios of 3.0−3.5, over a broader range of excitation amplitudes.

For HASTE experiments performed with a 2-ms excitation period, the results lie between those of CID and DCID. HASTE offers a maximum CID efficiency of 40%, while reaching a maximum a_4/b_4 ratio of 2.3. Like conventional CID, HASTE demonstrates a dependence on excitation amplitude that shows a relatively narrow optimal operation window for dissociation. The fragmentation efficiencies obtained in these experiments for DCID of leucine enkephalin are well below "ideal" limits for routine analyses, but previous experiments have shown that DCID can yield higher fragmentation efficiencies if performed at larger pseudopotential well depths, such as $q_z = 0.45$. The present experiments were performed at $q_z = 0.29$—to be consistent with previous efforts in the literature—and thus provide a larger dynamic mass range in which ions can be recaptured. Since

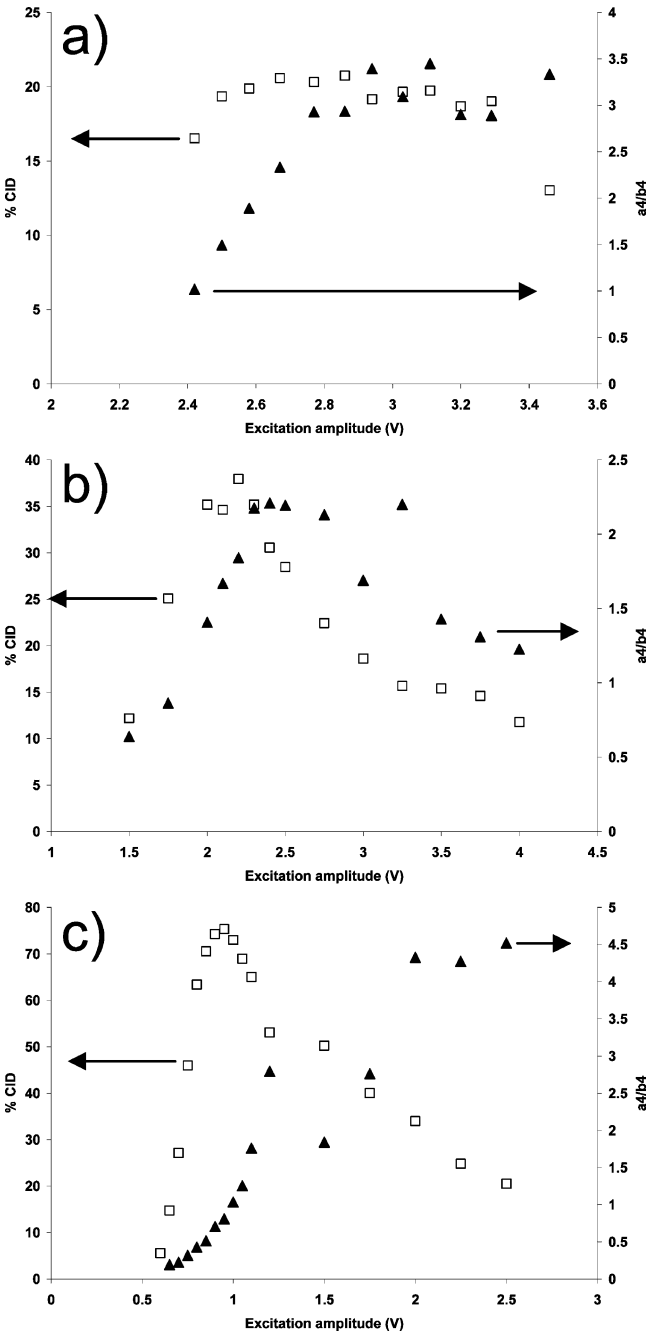

Figure 2. Percent CID efficiency (□) for the fragmentation of leucine enkephalin (YGGFL) by (a) DCID; (b) HASTE, 2-ms fragmentation; and (c) conventional CID, 30-ms fragmentation combined with the ratio of a_4/b_4 ions (▲) produced. Excitation performed at an excitation frequency of 81 kHz, $q_z = 0.29$.

fragmentation in DCID occurs during the mass acquisition scan, the q_z at which the excitation is performed also determines the LMCO for a given mass-to-charge ratio being analyzed. The lower CID efficiencies observed with both HASTE and DCID relative to conventional CID can, in part, be explained by an increase in the proportion of resonance ejection of the precursor ions caused by the higher excitation amplitudes.

To demonstrate that DCID also works for larger molecules and in both positive and negative modes, the mass spectra of substance P and oxidized insulin α-chain are also presented. Figures 3 and 4 demonstrate that DCID mass spectra produce

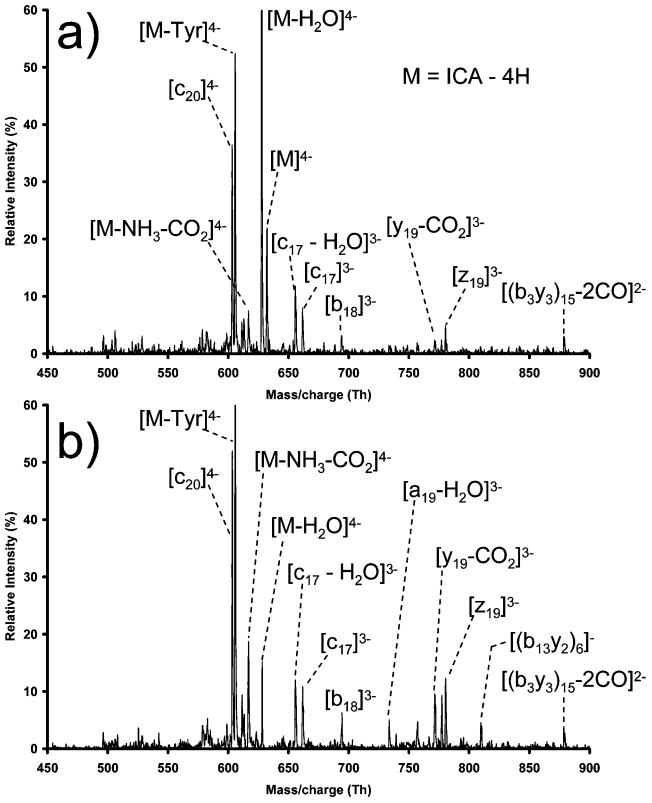

Figure 3. Comparison between (a) DCID for the 4− anion of oxidized insulin α-chain (ICA) at 2.69 V_{pp} and (b) conventional CID (30-ms fragmentation, 0.80 V_{pp}). Fragmentation performed at excitation frequency of 81 kHz, $q_z = 0.29$ and an average of 40 scans. Total analysis time is 10% shorter using DCID.

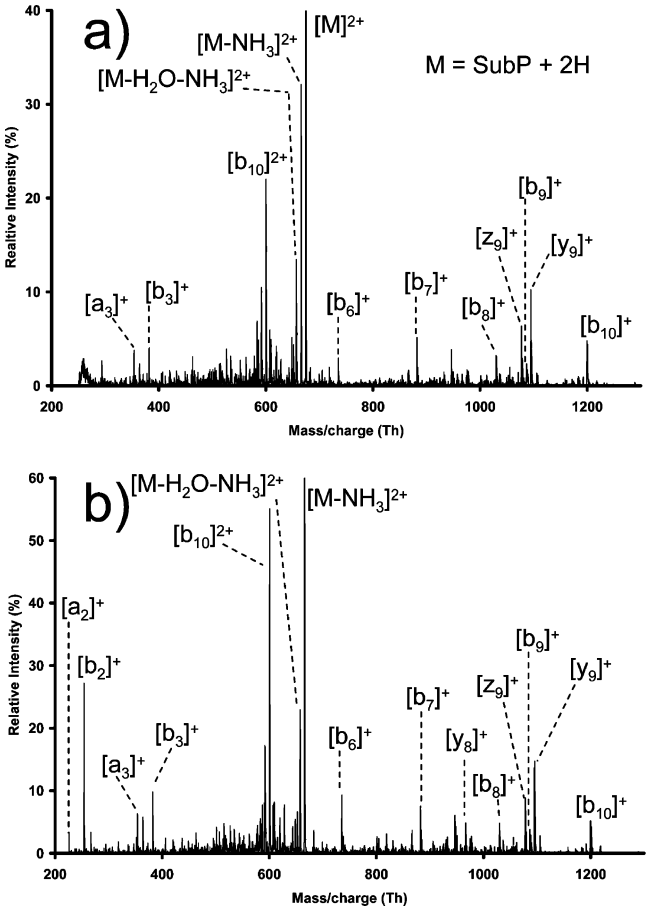

Figure 4. Comparison between (a) DCID for the 2+ ion of substance P (SubP) at 3.46 V_{pp} and (b) conventional CID (30-ms fragmentation, 1.25 V_{pp}). Fragmentation performed at excitation frequency of 81 kHz, $q_z = 0.29$ and an average of 30 scans. The time saving obtained by performing DCID is 12% for the analysis of substance P. The b_2^+ ion is not visible in the DCID spectrum because the application of resonance ejection to allow mass range extension causes the LMCO to be ~258 Th, slightly higher than b_2^+, which has a mass/charge ratio of 254 Th.

fragmentation patterns that are similar to those obtained using conventional CID, with the exception that more of the precursor ions tend to be left intact and that a mass peak representing the loss of water is usually more abundant than in conventional CID. The tandem mass spectra obtained using DCID for both peptides also matches product spectra previously published for oxidized insulin α-chain[40,41] and substance P.[42] The poor fragmentation efficiencies can be explained by the fact that the ions are excited only for a very brief period and that not all of ions received a sufficient amount of energy to induce dissociation. Smaller CID efficiencies were also observed in previous work using short excitation periods.[33,34] Figure 3 shows that DCID can indeed be used to obtain an adequate fingerprint product ion spectrum in which the distribution of product ions is qualitatively similar to conventional CID. The time savings for DCID are a modest 10% in this particular experiment, which is mostly limited by the very long isolation periods used by the Bruker software.

Using DCID, the lowest observable mass/charge ratio is also limited by the resonance ejection frequency. Therefore, it is always slightly higher than the LMCO for conventional CID performed at the same q_z. The difference in LMCO becomes even more important when mass range extension is performed, because the frequency used for the resonance ejection will be lower. This slight disadvantage of DCID over HASTE and conventional CID can be observed in the spectra of substance P shown in Figure 4, wherein the a_2^+ and the b_2^+ ions are only observed in the conventional CID spectrum (Figure 4b). In general, tandem mass spectra of peptides obtained with DCID are of good quality and greatly resemble those obtained for conventional CID and offer the advantage of a reduction in analysis time. However, HASTE spectra (not shown) and DCID spectra for all three peptides are very similar and tend to show stronger preference for H_2O loss than does conventional CID, as mentioned previously. The amplitudes of the excitation energy required to perform a HASTE experiment (2-ms excitation) were comparable with the excitation energies used to perform DCID for all three peptides studied. This indicates that with both methods the period over which the ions accumulate energy is relatively similar.

One of the main benefits of DCID over conventional CID is the reduction in analysis times. The percentage of time saved will vary depending on the scan rate and other acquisition parameters, but DCID is the only fragmentation method that does not add any time to the scan function of a single MS experiment (see Figure 1), other than the isolation period. Another advantage of DCID over other methods with short on-resonance excitation,

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such as HASTE[34] or "fast excitation",[33] is that there is no need to tune the excitation frequency. If the ions are perturbed by higher-order fields or by space-charging, they will always have to cross the "plane" of the applied excitation frequency. In other words, DCID obviates the need for resonance tuning because the ions are guaranteed to coincide with the excitation frequency at some point during the mass instability scan.

CONCLUSION

Peptides up to 2.5 kDa can easily be characterized using DCID with product ion spectra very similar to those obtained with conventional CID. Therefore, DCID spectra can be interpreted or analyzed using existing methods or databases. Exciting the ions at a common stability parameter of q_z of 0.29 allows the collection of fragment ions with a mass-to-charge ratio of roughly 40% of the precursor ion. This LMCO is larger than in conventional CID and arises from the fact that the resonance ejection waveform is being applied simultaneously, thus reducing the available trapping range. Ideally, the DCID method will be adapted to allow the collection of ions at of much lower cutoff values, in a manner similar to "pulsed Q" activation.[34,35] Cunningham and co-workers[34] recently showed that it is possible to rapidly reduce the drive rf amplitude immediately after excitation in order to recapture low-mass product ions. The fragmentation efficiency of DCID could presumably be improved by applying an excitation waveform during mass acquisition that is composed of two or more frequencies simultaneously.[37] Two-frequency DCID has been shown to be more efficient and energetic than single-frequency DCID for small organics such as *n*-butylbenzene. DCID is likely to be most advantageous when combined with fast or complex liquid separation methods, such as UPLC, monolithic HPLC, or 2D-LC, by increasing the number of tandem mass spectra that can be acquired in a given period. If combined with 2D-LC separations in a proteomics-type separation, DCID could allow at least 10% more data points, or potentially 10% more peptide hits than conventional CID. By shortening the isolation time of the precursor ions, time savings of 25% could be realized.

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