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Enabling MALDI-FTICR-MS/MS for High-Performance Proteomics through Combination of Infrared and Collisional Activation

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Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) is a central tool for proteomic analysis, yet the singly protonated tryptic peptide ions produced by MALDI are significantly more difficult to dissociate for tandem mass spectrometry (MS/MS) than the corresponding multiply protonated ions. In order to overcome this limitation, current proteomic approaches using MALDI-MS/MS involve high-energy collision-induced dissociation (CID). Unfortunately, the use of high-energy CID complicates product ion spectra with a significant proportion of irrelevant fragments while also reducing mass accuracy and mass resolution. In order to address the lack of a high-resolution, high mass accuracy MALDI-MS/MS platform for proteomics, Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) and a recently developed MS/MS technique termed CIRCA (for combination of infrared and collisional activation) have been applied to proteomic analysis. Here, CIRCA is shown to be suitable for dissociating singly protonated tryptic peptides, providing greater sequence coverage than either CID or infrared multiphoton dissociation (IRMPD) alone. Furthermore, the CIRCA fragmentation spectra are of sufficient quality to allow protein identification based on the MS/MS spectra alone or in concert with the peptide mass fingerprint (PMF). This is accomplished without compromising mass accuracy or mass resolution. As a result, CIRCA serves to enable MALDI-FTICR-MS/MS for high-performance proteomics experiments.

The dissociation of peptide ions in tandem mass spectrometry (MS/MS) is currently a significant focus of both theoretical and practical research. For example, many recent efforts have been directed toward improving the quality of peptide ion tandem mass spectra through chemical modification[1−4] and instrumental methods.[5−15] These areas of research have been complimented by fundamental mechanistic investigations on peptide ion fragmentation,[16−25] as well as descriptive characterization of large peptide ion MS/MS data sets.[26−30] Much of the motivation for these endeavors can be attributed to ongoing growth and develop-

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ment in the field of mass spectrometry (MS) based proteomics. In this regard the MS/MS dissociation of tryptic peptide ions has been emphasized, as these are the most common analytes of proteomic research. Trypsin is the preferred protease for applications in proteomics due to high activity and high fidelity for peptide bond hydrolysis C-terminal of arginine or lysine residues (unless the next residue is proline). The activity and specificity of tryptic cleavage produces peptides with a size distribution favorable for analysis and sequence determination by MS, and these peptides are efficiently ionized by both electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) due to the presence of an amino acid residue with high gas-phase proton affinity.

Importantly, the presence of a C-terminal basic residue dictates much of the gas-phase ion chemistry governing MS/MS fragmentation of tryptic peptide ions. As predicted by the mobile proton model of fragmentation,[16] peptide ions with a greater number of charge-carrying protons than basic residues are capable of undergoing fragmentation at multiple sites through a number of distinct charge-directed dissociation pathways that occur with similar activation energies. By contrast, charge-directed fragmentation becomes less energetically accessible when the number of basic residues is greater than or equal to the number of charge-carrying protons. In these cases, all charge-carrying protons are captured by the basic residues, and charge-directed fragmentation pathways become significantly elevated in activation energy. Under such circumstances, any available charge-remote fragmentation pathways out-compete the charge-directed channels. Because charge-remote fragmentation usually involves only a single site along the peptide backbone, such product ions provide very limited information. On the basis of these considerations, it becomes clear that singly protonated tryptic peptide ions represent particularly challenging targets for low-energy MS/MS.

In proteomics, MS/MS of tryptic peptides is usually accomplished using collision-induced dissociation (CID) of multiply protonated ions generated by ESI. Alternatively, the singly protonated ions generated by MALDI are fragmented via CID in a tandem time-of-flight (TOF/TOF) instrument. In the latter case, ions are accelerated through relatively high potentials (i.e., hundreds of eV) prior to collision, allowing singly protonated tryptic peptides to be probed by MS/MS despite their inherent resistance to fragmentation. Typically, a MALDI-TOF instrument is used for peptide mass fingerprinting (PMF) experiments, in which proteins separated by gel electrophoresis are digested with trypsin and identified on the basis of single-stage MS alone.[31−35] The use of a MALDI instrument capable of MS/MS compliments this approach, as the same instrument and same sample can be used to clarify any ambiguous protein assignments through the fragmentation of selected peptides. Unfortunately, the high-energy CID employed by MALDI-TOF/TOF introduces significant complications to the MS/MS analysis. One recent study of over 2400 curated MALDI-TOF/TOF tandem mass spectra found that internal and unassigned fragments accounted for nearly 40% of all observed product ions.[28] Although the mass accuracy and mass resolution of single-stage TOF analyzers has improved in recent years, another limitation of MALDI-TOF/TOF lies in the significantly diminished resolution and accuracy of the MS/MS spectra. These limitations notwithstanding, the use of MALDI for characterizing protein tryptic digests has several advantages, including more rapid analysis and low susceptibility to ion suppression as compared to ESI. In addition, offline MALDI-MS can be used to more comprehensively characterize peptides separated by liquid chromatography (LC), as the peaks are essentially "parked" for analysis and can be examined in a time frame independent of LC peak width.

In light of the foregoing discussion, a number of distinct advantages would be afforded by an MS instrument based on MALDI with the capability to provide informative MS/MS of singly protonated tryptic peptides without excessive collision energy and without compromising mass accuracy or resolution. At present, there is no single platform that meets all of these criteria. The coupling of MALDI to Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) has already proven exceptionally capable for proteomic analyses and should provide many of the desirable attributes discussed here.[36−38] This combination of ion source and mass analyzer affords the benefits of MALDI for peptide analysis while also furnishing high accuracy (mass errors of a few parts per million) and high resolution ($m/\Delta m > 10^5$ in broad-band mode).[39,40] With regards to MS/MS, both sustained off-resonance irradiation collision-induced dissociation (SORI-CID)[41−43] and infrared multiphoton dissociation (IRMPD)[44,45] have been performed in conjunction with MALDI-FTICR-MS. However, a typical SORI-CID experiment is capable of providing fragmentation energies of only about 10 eV,[46] and the energy of an IR photon at the 10.6 μm wavelength supplied by CO_2 lasers is 0.12 eV. Since these MS/MS techniques are intended to sample only the lowest energy dissociation pathways, they have not proven very useful for fragmentation of singly protonated tryptic peptides.

Recently, infrared (IR) irradiation with simultaneous SORI in the presence of collision gas was shown to significantly enhance dissociation of singly protonated model peptides analyzed by

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MALDI-FTICR-MS/MS.[47] This new MS/MS technique, referred to as CIRCA (for combination of infrared and collisional activation), yielded richer fragmentation spectra than could be achieved using IRMPD or SORI-CID alone. It was proposed that the observed improvements in fragmentation were principally attributable to CID of ions already residing in IR-excited vibrational states—a concept not dissimilar from previous descriptions of "thermally assisted IRMPD",[48] "collision-activated IRMPD",[49] and "thermally assisted CID"[50] performed in ion trap mass analyzers. Although CIRCA allowed improved fragmentation of simple model peptides as compared to SORI-CID or IRMPD alone, the question of whether CIRCA-enhanced dissociation translated to more realistic and more difficult tryptic peptides remained unaddressed. Even assuming some improvement in dissociation of these peptide ions, it was not clear whether the resulting MS/MS spectra would be of sufficient quality for practical applications in proteomics. Here, we have assessed the extent to which CIRCA can participate in the advancement of MALDI-FTICR-MS/MS as a platform for high-performance proteomic analysis. The extension of CIRCA to the interrogation of singly protonated peptides derived from tryptic proteolysis has thus been pursued with the goal of devising a MALDI-MS/MS approach for low-energy peptide dissociation with high-accuracy and high-resolution mass analysis.

EXPERIMENTAL SECTION

Preparation of Tryptic Peptides. Bovine serum albumin (BSA), bovine α-lactalbumin (BAL), and bovine lactoferrin (BLF) were obtained from Sigma (St. Louis, MO). Stock solutions of each protein were prepared at 10 μg/μL in 8 M urea with 100 mM NH_4HCO_3 (pH = 7.8). Aliquots of each protein (1 μL of the stock solution) were combined with 40 μL of 8 M urea/100 mM NH_4HCO_3 and 10 μL of 450 mM dithiothreitol in 50 mM NH_4HCO_3. Protein reduction was carried out by incubating the samples at 55 °C for 1 h. Each reduced protein solution was then treated with 10 μL of 500 mM iodoacetamide in 50 mM NH_4HCO_3 for alkylation. The mixtures were then held in the dark at ambient temperature for 1 h. Each sample was next diluted with 150 μL of deionized water to bring the urea concentration to <2 M. Trypsin was added to each preparation (1 μL of a 0.05 μg/μL solution in 50 mM NH_4HCO_3), and the samples were incubated at 37 °C for approximately 12 h. A 10 μL aliquot of each tryptic digest was desalted by solid-phase extraction with C18 ZipTips (Millipore, Billerica, MA), and the purified tryptic peptides were eluted in 10 μL of 50% acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA).

Mass Spectrometry. A matrix solution of 2,5-dihydroxybenzoic acid (DHB; Sigma) was prepared at a concentration of 50 μg/μL in 50% ACN with 0.1% TFA. Desalted peptide preparations were spotted on a stainless steel MALDI target with an equal volume of matrix (typically, 1 μL each) such that digest from approximately 1 pmol of protein was deposited. All MS analyses were conducted using a 7.0 T FTICR-MS instrument employing an external MALDI source with hexapole ion accumulation (Varian IonSpec ProMALDI, Lake Forest, CA). Typically, ions produced by five MALDI laser pulses (Nd:YAG, 355 nm, 5 ns pulse width) were accumulated and vibrationally cooled using a controlled leak of nitrogen into the hexapole chamber. Ions were then injected into the ICR cell via an rf-only quadrupole ion guide. Following ion acceleration, mass spectra were acquired over the m/z range of 220−4500 (1024 k transient data points, 1 MHz ADC rate). All single-stage mass spectra were internally calibrated using the InCAS technique.[51,52] Specifically, analyte and standard ions produced in separate MALDI events were accumulated in the hexapole, and the combined population of ions was transferred to the ICR cell and mass analyzed simultaneously. Bradykinin fragment peptide 1−7, angiotensin II, $P_{14}R$, human adrenocorticotropic hormone fragment peptide 18−39, and oxidized B chain of bovine insulin (all obtained from Sigma) served as calibrant peptides. A solution containing each peptide at a concentration of 1 μM was prepared in 50% ACN/0.1% TFA, and 1 μL of this mixture was cospotted with the DHB matrix solution as described above.

For tandem mass spectrometry, precursor ions of interest were isolated in the ICR cell by stored waveform inverse Fourier transform (SWIFT) ejection of the unwanted ions. Ion activation was then performed by either SORI-CID, IRMPD, or CIRCA as described previously.[47] SORI excitation was performed at amplitudes of 3.0−8.0 $V_{(b-p)}$ with frequency offsets of +1.0 kHz relative to the precursor ion cyclotron frequency. During each 1.0 s SORI event, the pressure in the ICR cell was raised to approximately 10^{-5} Torr by a brief pulse of nitrogen. The analyzer cell pressure was allowed to return to approximately 10^{-10} Torr prior to ion excitation and signal acquisition. A CO_2 laser (10.6 μm, 20 W maximum power, Parallax, Waltham, MA) provided photons for IRMPD and CIRCA experiments. The IR laser setup has been described in detail elsewhere.[53−57] IR laser pulse widths ranged from 1.5 to 3.0 s, and the laser power was held at 20 W. The SORI amplitude and IR pulse width were each adjusted to produce maximum fragmentation without undue loss of signal. When comparing IRMPD, SORI-CID, and CIRCA fragmentation of a given tryptic peptide, the IRMPD and SORI-CID parameters were individually optimized and the same parameters were combined in the corresponding CIRCA experiment by triggering the IR laser during the SORI pulse. For a given MS/MS experiment, up to 20 transients were acquired and summed to produce the final spectrum. All comparisons between IRMPD, SORI-CID, and CIRCA were drawn on the basis of an equal number of summed scans. The mass calibrations for all tandem mass spectra were internally corrected using the putative measured mass of the precursor ion from the corresponding internally calibrated single-stage mass spectra.

Data Processing and Proteomic Database Searching. Internal mass calibration and internal mass correction of all spectra was performed using the IonSpec Omega software according to

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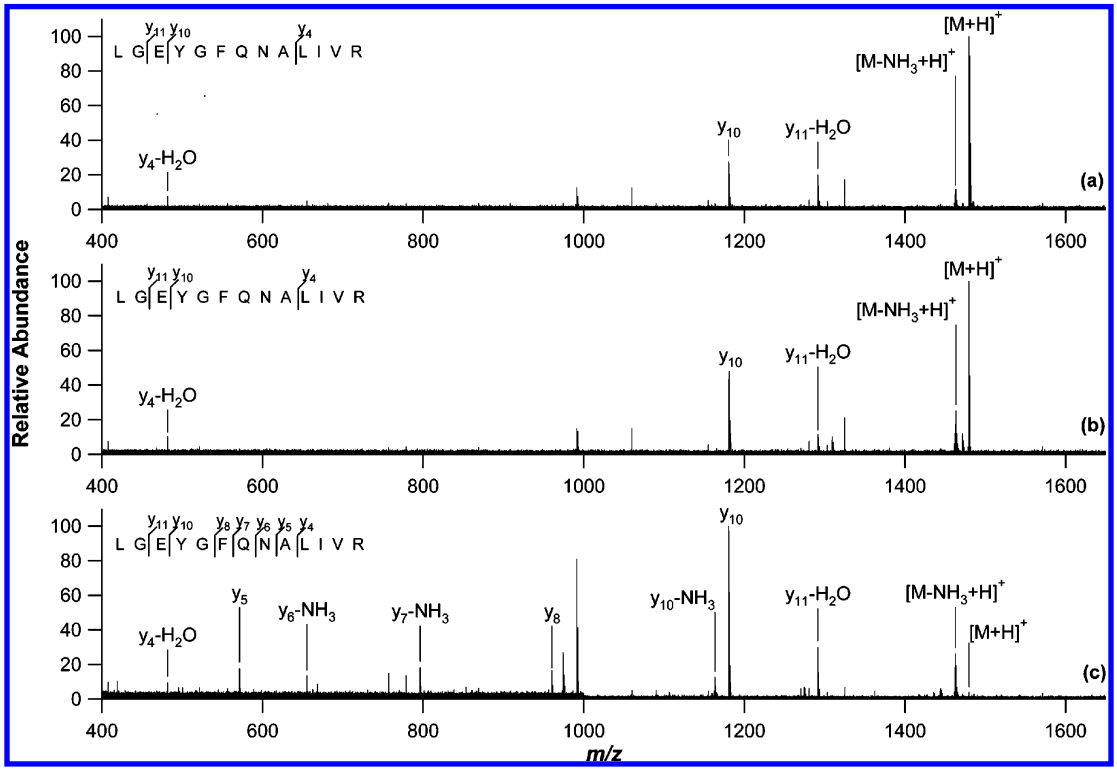

Figure 1. MALDI-FTICR-MS/MS of a BSA tryptic peptide (m/z = 1479.7901) with IRMPD (a), SORI-CID (b), and CIRCA (c). The IR pulse width in (a) and (c) was 3.0 s; the SORI amplitude in (b) and (c) was 8.0 $V_{(b-p)}$. Below m/z 1000, the abundance scales have been expanded 2-fold in (a) and (b) and 4-fold in (c).

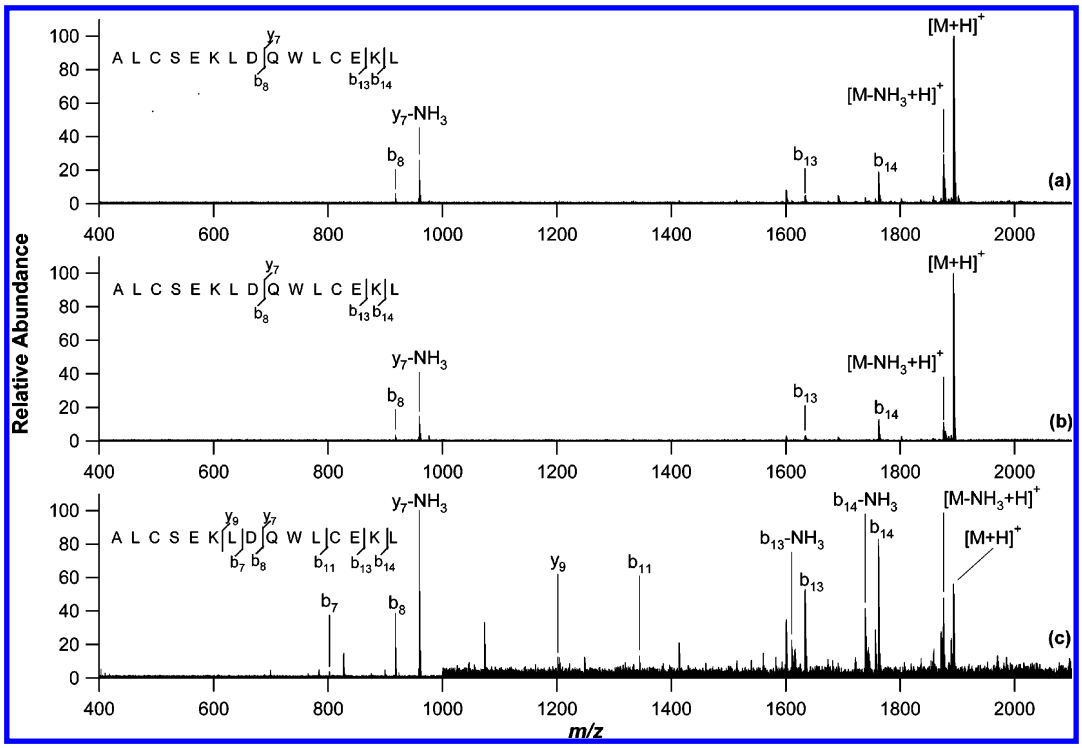

Figure 2. MALDI-FTICR-MS/MS of a BAL tryptic peptide (m/z = 1892.9200) with IRMPD (a), SORI-CID (b), and CIRCA (c). The IR pulse width in (a) and (c) was 2.0 s; the SORI amplitude in (b) and (c) was 8.0 $V_{(b-p)}$. In (c), the abundance scale has been expanded 4-fold above m/z 1000 due to depletion of the higher mass ions.

standard FTICR-MS calibration relationships.[39,40] Calibrated spectra were further processed using the IonSpec PeakHunter software, and the resulting thresholded, monoisotopic peak lists were exported as text format files. Peak lists were further processed using an in-house algorithm called Mass Sieve that was programmed and implemented using the IGOR Pro software package (version 6.0, Wave Metrics, Lake Oswego, OR). This program was used to exclude any interfering, nonpeptide masses from the peak lists on the basis of accurately measured monoisotopic mass, as has been described in detail previously.[38,58] It should

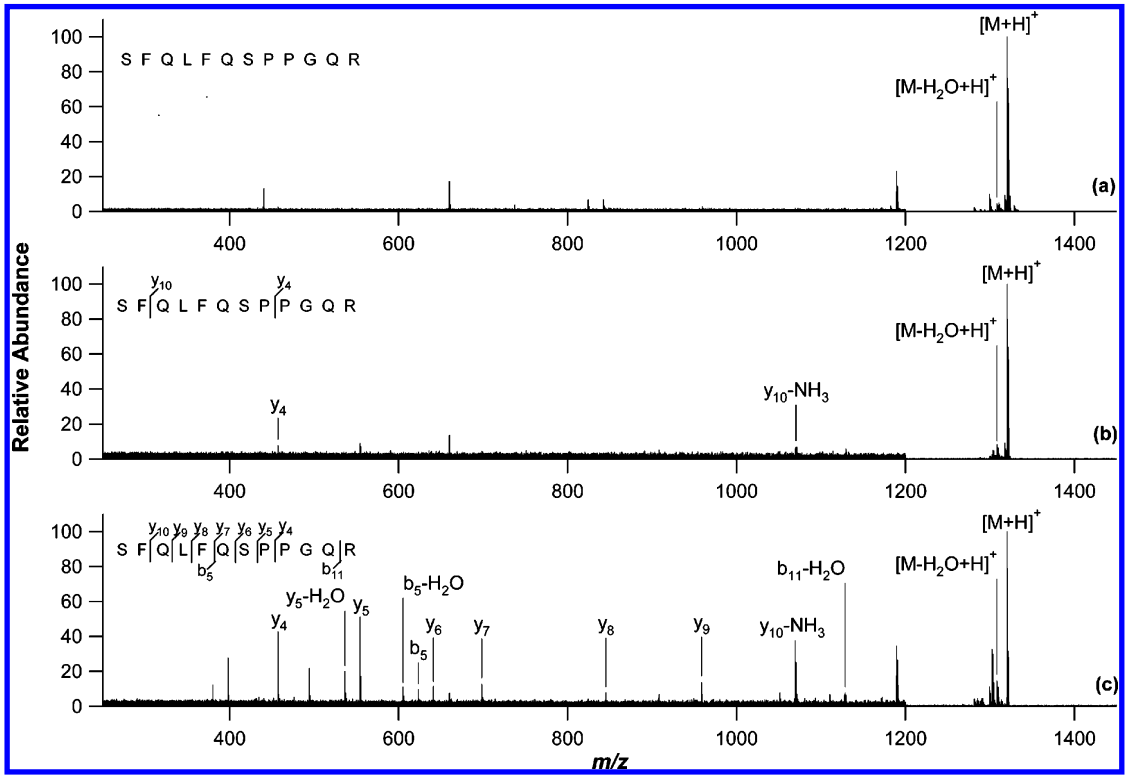

Figure 3. MALDI-FTICR-MS/MS of a BLF tryptic peptide (m/z = 1320.6681) with IRMPD (a), SORI-CID (b), and CIRCA (c). The IR pulse width in (a) and (c) was 2.0 s; the SORI amplitude in (b) and (c) was 5.5 $V_{(b-p)}$. Below m/z 1200, the abundance scales have been expanded 10-fold in (a) and (b) and 5-fold in (c).

be noted that the software referred to herein was developed in this laboratory and should not be mistaken for an unrelated proteomics program that has more recently assumed the name "MassSieve."[59] Internal standard masses and common contaminant peptide masses (i.e., trypsin autolysis peptides and keratin peptides, if present) were also screened from the peak lists at this stage with a mass error tolerance of 5 ppm. The refined peak lists containing monoisotopic $[M + H]^+$ masses were saved in text file format.

Proteomic database queries were performed using the Mascot MS/MS ions search (www.matrixscience.com).[60] The Mass Spectrometry Protein Identification database (MSDB) was searched within the taxonomic category of "other mammalia." To reflect the alkylation with iodoacetamide, carbamidomethylation was specified as a fixed modification of cysteine residues. Usually, one missed tryptic cleavage was tolerated; however, in one case this was relaxed to two tryptic misses in order to account for all major peptide signals. The mass error tolerance was set at 5 ppm for precursor ions and at 20 mDa for fragment ions (i.e., 10 ppm at m/z 2000). The absolute error tolerance was applied because Mascot does not currently support relative error tolerance for product ions. The "instrument type" parameter, which determines the fragment ion series to be recognized by the search algorithm, was set to MALDI-TOF-TOF. This selection was made because it was the most inclusive in searching for fragment ion series. For each data set submitted for protein identification, two report types were generated: a peptide summary report (which takes into account only MS/MS fragment ions), and a protein summary report (which takes into account peptide ions from the single-stage MS of the protein digest, in addition to the MS/MS fragment ions).

RESULTS AND DISCUSSION

Comparison of IRMPD, SORI-CID, and CIRCA. As an initial step toward determining the applicability of CIRCA to peptide ions of proteomic relevance, the dissociation of several tryptic peptides was performed with IRMPD, SORI-CID, and CIRCA under identical conditions for direct comparison. Each assessment was based on the same number of spectral scans, and the parameters used in the individual SORI-CID and IRMPD experiments were not altered for the corresponding CIRCA experiments.

The IRMPD, SORI-CID, and CIRCA tandem mass spectra for an abundant tryptic peptide of BSA are shown in Figure 1. The high degree of similarity between the IRMPD and SORI-CID spectra is immediately evident, with each providing three y-series fragment ions. By comparison, the CIRCA tandem mass spectrum provided richer peptide sequence information, with seven y-type ions observed. Notably, the CIRCA experiment resulted in the almost complete depletion of the precursor ion, further illustrating increased energy deposition with CIRCA as opposed to IRMPD or SORI-CID alone. In each of the MS/MS experiments, the y_{10} ion was observed as an abundant product. This result was not unexpected, as this cleavage occurred on the C-terminal side of

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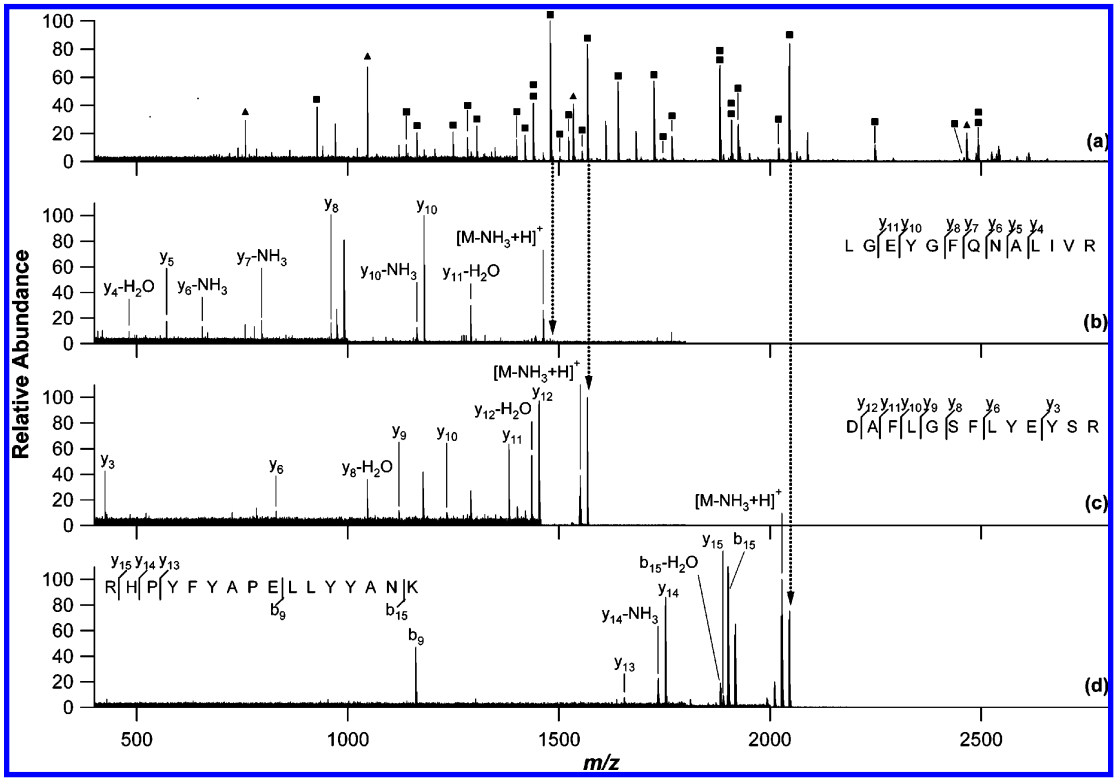

Figure 4. MALDI-FTICR-MS PMF of BSA (a) and CIRCA MS/MS of BSA tryptic peptides at *m*/*z* 1479.7901 (a), 1567.7377 (b), and 2045.0244 (c). In (a), masses matched to BSA tryptic peptides within 5 ppm are labeled with squares, and internal calibrant peptides introduced by InCAS are labeled with triangles. The abundance scales have been expanded for clarity as follows: 4-fold below *m*/*z* 1400 in (a); 4-fold below *m*/*z* 1000 in (b); 10-fold below *m*/*z* 1450 in (c); 5-fold below *m*/*z* 2000 in (d).

an acidic residue and is due to a lower energy, charge-remote fragmentation process. This phenomenon is generally known as the "aspartic acid effect", although in this case the acidic residue involved was glutamic acid.[16] In such cases, the lower energy charge-remote processes often lead to a single predominant fragment type (such as the y_{10} ion in this example) while averting the higher energy charge-directed pathways that would lead to other informative sequence ions. Thus, whereas SORI-CID and IRMPD were each only able to provide two sequence ions in addition to the lowest energy channel, CIRCA yielded six additional sequence fragments resulting from charge-directed mechanisms despite the presence of the competing charge-remote process. This capability was further demonstrated in the MS/MS analysis a major tryptic peptide derived from BAL (Figure 2). In contrast to the previous example of a fully tryptic peptide (i.e., a peptide with no missed tryptic cleavage sites), this peptide ion was in possession of two basic residues (both lysine, due to two missed tryptic cleavages) and C-terminated by a leucine residue rather than a lysine or arginine (since this peptide was derived from the C-terminus of the intact protein). In common with the previous example, however, was the potential for energetically preferred fragmentation sites (in this case, one aspartic acid and two glutamic acid residues) that could preclude good sequence coverage across the peptide backbone. Again, IRMPD and SORI-CID were found to provide essentially the same fragment ion spectra, with each resulting in one y-type and three b-type product ions. Two of these four cleavages occurred C-terminal to acidic residues (b_8 and b_{13}). Thus, half of the fragment ions observed by IRMPD and SORI-CID could be attributed to charge-remote dissociation chemistry. The interrogation of this peptide ion by CIRCA resulted in greater diminution of the precursor ion intensity and produced two y-type and five b-type fragment ions. Only two of these were attributable to charge-remote fragmentation mechanisms. The MS/MS analysis of these two tryptic peptides served to underscore the capability of CIRCA to provide sufficient energy for accessing charge-directed fragmentation channels in addition to the lowest energy charge-remote pathways. Moreover, this was found to hold true for charge-directed dissociation products not accessible by either IRMPD or SORI-CID alone.

A characteristic fully tryptic fragment of BLF was also probed with each of the three MS/MS activation methods. The comparison provided in Figure 3 serves as another example of the enhanced fragment ion yield afforded by CIRCA. Every attempt at IRMPD of this ion failed to produce any detectable sequence ions, despite the high abundance of precursor ions isolated for MS/MS. Although IRMPD of the previously discussed BSA and BAL tryptic peptides yielded at least a few useful fragment ions, it should be noted that the masses of those ions were significantly higher (with nominal m/z values of 1480 and 1893, respectively). The failure of IRMPD to produce any detectable b or y ions from the BLF precursor ion with a nominal m/z value of 1321 was most likely attributable to lower molar mass, which has been noted in the past to result in reduced IRMPD efficiency. Such effects have been previously observed in this laboratory during experiments involving IRMPD of oligosaccharides and CIRCA of model nontryptic peptides; moreover, dramatic decreases in fragmentation efficiency have been observed over relatively narrow range

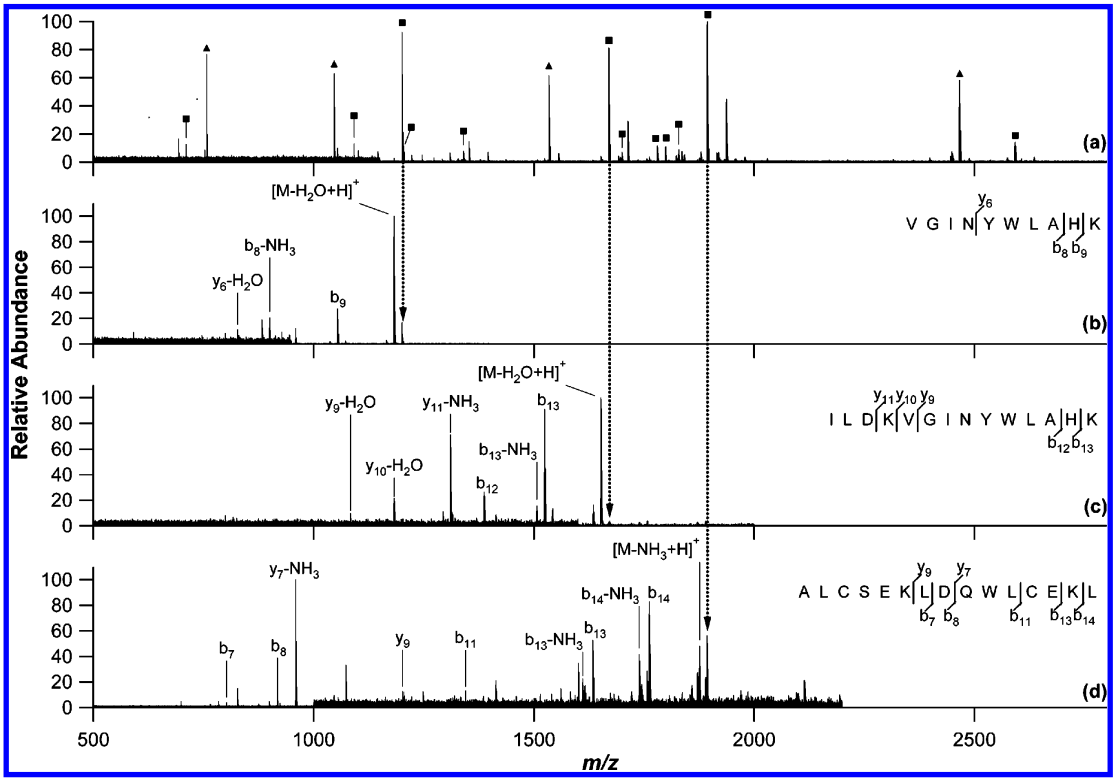

Figure 5. MALDI-FTICR-MS PMF of BAL (a) and CIRCA MS/MS of BAL tryptic peptides at *m/z* 1200.6487 (a), 1669.9382 (b), and 1892.9200 (c). In (a), masses matched to BAL tryptic peptides within 5 ppm are labeled with squares, and internal calibrant peptides introduced by InCAS are labeled with triangles. The abundance scales have been expanded for clarity as follows: 5-fold below *m/z* 1150 in (a); 10-fold below *m/z* 950 in (b); 4-fold below *m/z* 1600 in (c); 4-fold above *m/z* 1000 in (d).

of molar mass reduction.[47,56] These previous findings are consistent with the difference in IRMPD efficiency presently observed between *m/z* 1480 and *m/z* 1321 precursor ions. On the basis of these observations, IRMPD would not appear to be capable of dissociating singly protonated tryptic peptide ions with masses of less than approximately 1400 Da. SORI-CID of the BLF tryptic peptide also produced relatively little information, with only two y-type ions detected. Strikingly, CIRCA dissociation of this same peptide ion yielded seven y-type and two b-type fragment ions, thereby providing ostensibly complete elaboration of the peptide sequence. In accordance with these results, it may be soundly concluded that the previously demonstrated advantages of CIRCA are directly extensible to singly protonated tryptic peptide ions, which represent particularly challenging targets for low-energy dissociation.

Protein Identification Using MS/MS with CIRCA. With the facility of CIRCA for dissociation of tryptic peptides well established, the suitability of these tandem mass spectra for protein identification was next investigated. The PMF of BSA is shown in Figure 4a, and the CIRCA MS/MS spectra of the three most abundant tryptic peptides are shown in Figure 4b–d. The PMF ultimately yielded 30 masses matching predicted BSA tryptic peptides to within 5 ppm mass error. Interrogating each of the three most abundant peptide ions with CIRCA provided a total of 17 y-series cleavages and two b-series cleavages. Despite the presence of two acidic residues (one aspartic acid and one glutamic acid), the tryptic peptide with a nominal *m/z* value of 1568 produced seven y-type ions (Figure 4c). The two fragments arising from the aspartic acid effect (y_3 and y_{12}) were among the more abundant products; however, several other sequence ions were clearly identified. Charge-remote fragmentation also played a significant role in the dissociation of the peptide ion at *m/z* 2045 (Figure 4d), with one of the most abundant product ions (b_9) occurring on the C-terminal side of the glutamic acid residue. Nonetheless, four additional peptide bond cleavages were observed with both b and y fragments represented.

The PMF of BAL tryptic peptides is provided in Figure 5a. Twelve tryptic peptides were matched to the target protein (±5 ppm mass error tolerance), and three of these were isolated and probed with CIRCA (Figure 5b–d). Although the peptide ion at *m/z* 1201 was among the most abundant species derived from this protein, the CIRCA dissociation of this ion produced only three sequence cleavages, reiterating the apparent mass dependence IR-dependent activation techniques for peptide dissociation (Figure 5b). It appears unlikely that this observation is unique to a particular peptide, as we have previously noted this behavior using other model peptides.[47] The BAL tryptic peptide at *m/z* 1670 contained the same segment of sequence that constituted the *m/z* 1201 peptide, with the addition of four additional amino acid residues at the N-terminus due to a tryptic miss at the internal lysine residue. For this peptide ion, five total sequence fragments were discerned (three y-type and two b-type). Two of these (b_{12}, b_{13}) were equivalent to the cleavages seen in the CIRCA MS/MS spectrum of the *m/z* 1201 peptide ion (b_8, b_9), and the remaining three provided new sequence information. Overall, 15 sequence cleavages were obtained for the three interrogated BAL tryptic peptides.

BLF served as a third model for evaluating the usefulness of CIRCA MS/MS in protein identification. Of the 26 tryptic peptides mapped to BLF with 5 ppm mass error or less (Figure 6a), three

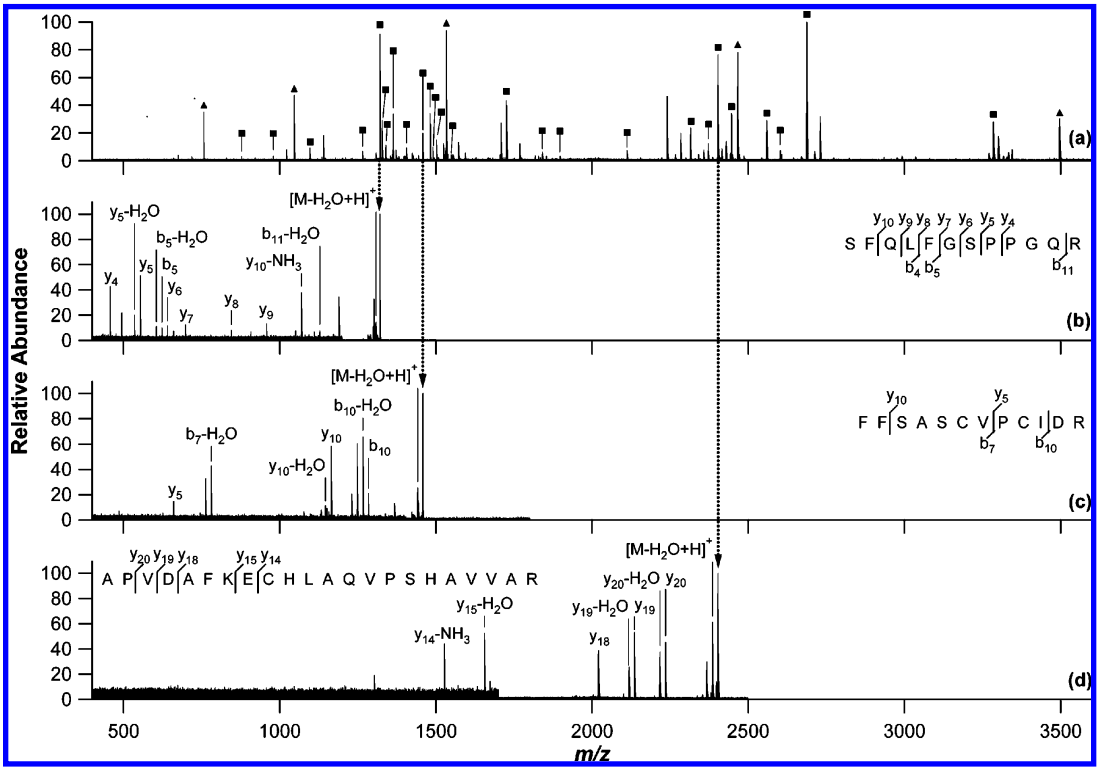

Figure 6. MALDI-FTICR-MS PMF of BLF (a) and CIRCA MS/MS of BLF tryptic peptides at *m*/*z* 1320.6681 (a), 1458.6521 (b), and 2402.2371 (c). In (a), masses matched to BLF tryptic peptides within 5 ppm are labeled with squares, and internal calibrant peptides introduced by InCAS are labeled with triangles. The abundance scales have been expanded for clarity as follows: 5-fold below *m*/*z* 1200 in (b); 2-fold below *m*/*z* 1400 in (c); 5-fold below *m*/*z* 1700 in (d).

were isolated and subjected to CIRCA for dissociation (Figure 6b–d). Those MS/MS experiments produced 19 sequence ion cleavages. Five of these were of the b-type, with y-type fragmentation accounting for the remainder. The singly protonated tryptic peptides of BLF successfully interrogated with CIRCA ranged from *m*/*z* 1321 to *m*/*z* 2402, thus highlighting the broad *m*/*z* range over which CIRCA maintains effectiveness for dissociation of singly protonated tryptic peptides.

The combination PMF and MS/MS for corroboration of selected peptide assignments is known to provide high-confidence protein identification. With the use of CIRCA this dimension of proteomic analysis was attained using MALDI-FTICR-MS/MS, thus allowing for orthogonal corroboration of protein identity while providing mass measurements of high resolution and high accuracy. As depicted in Figure 7a–c, each of the proteins considered here was correctly identified with a high level of statistical confidence based on augmentation of the PMF with CIRCA dissociated peptides shown in Figures 4–6. The PMFs provided sequence coverage of 60% for BSA and BAL, and 44% for BLF. In addition, the CIRCA dissociation of the three peptides shown in Figure 4b–d constituted 6% coverage for BSA; CIRCA dissociation of the two peptides shown in Figure 5, parts c and d, constituted 20% sequence coverage for BAL, and CIRCA dissociation of the three peptides shown in Figure 6b–d provided 6% sequence coverage of BLF. Although a number of additional, statistically significant hits can be seen the Mowse score distributions resulting from these queries, all of the additional matching proteins were either due to redundant indexing of the same protein in the database or due to close analogues of the correct protein (e.g., the hit for sheep albumin seen Figure 7a). In addition to effectively supplementing PMF data, the MS/MS dissociation spectra achieved with CIRCA allowed each of the proteins to be unambiguously assigned without any additional information (Figure 7, parts d and e). This suggests a role for CIRCA not only in the confirmation of peptides observed in PMF, but also in more "shotgun" oriented approaches with offline coupling to separation of peptides produced by tryptic digestion of complex protein mixtures. Due to the availability of very few sequence ions in the SORI-CID and IRMPD spectra, no protein identifications could be made using these techniques. Finally, it should be pointed out that these identifications were achieved with high mass accuracy in both MS dimensions. The root-mean-square mass error for peptides measured in single-stage MS (i.e., PMF) did not exceed 2 ppm for any protein identification, and the majority of CIRCA fragment ion masses were measured with well under 5 ppm error (Table 1).

CONCLUSIONS

Due to the presence of strongly basic C-terminal residues and in light of the mobile proton model, singly protonated tryptic peptides have been relatively inaccessible to tandem mass spectrometry using the low-energy dissociation techniques commonly employed in conjunction with FTICR-MS. This study has demonstrated that the advantages associated with the CIRCA tandem MS technique are directly portable to the proteomic analysis of realistic tryptic peptides as singly protonated ions. In addition to surpassing the performance of both CID and IRMPD, the MS/MS spectra obtained using CIRCA are of sufficient quality to allow protein identification directly or in concert with other peptide mass data. Although the fragment ion series produced

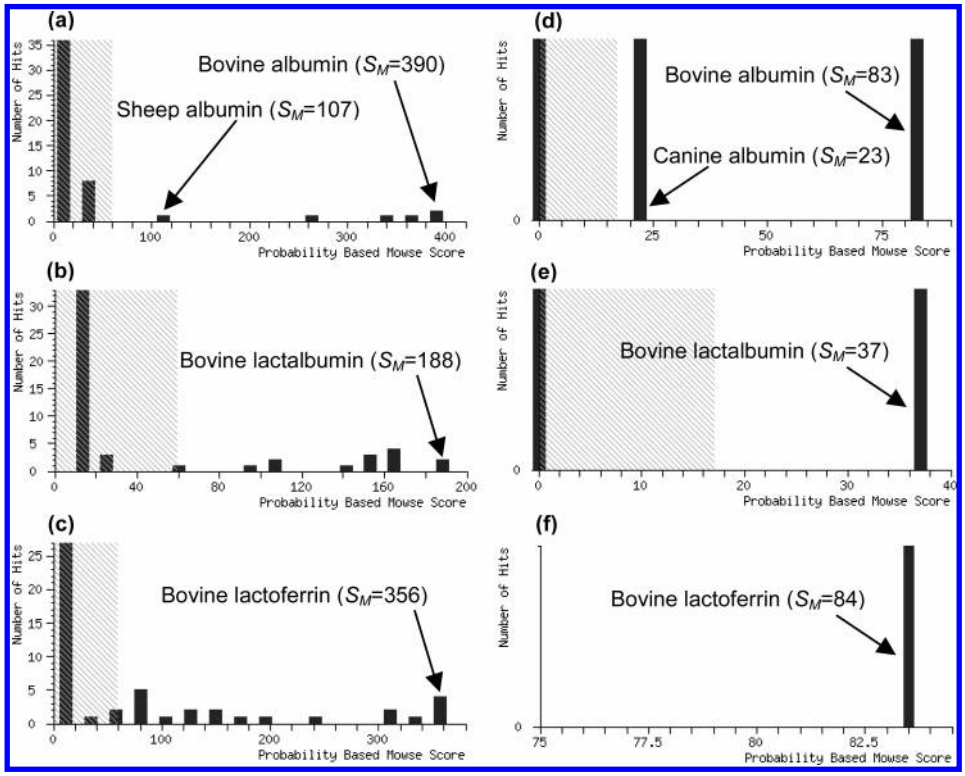

Figure 7. Mowse score (S_M) distributions reported by Mascot for MS/MS ion searches using CIRCA fragmentation spectra. The scoring results were based on PMF data supplemented with the CIRCA fragmentation data (a−c) and separately on the CIRCA fragmentation data alone (d and e). Protein matches falling beyond the shaded region are significant at $p < 0.05$. In (a−c), this significance cutoff was at $S_M = 59$; in (d and f), the significance cutoff was at $S_M = 17$. Note that the *x*-axes are scaled differently among the plots.

Table 1. Assigned Fragment Ions and Corresponding Mass Errors Resulting from CIRCA Dissociation of Selected Tryptic Peptides

peptide	ion type	obsd m/z (Da)	theor m/z (Da)	error (ppm)
BSA: ^{421}LGEYGFQNALIVR^{433}	$y_{11}-H_2O$	1291.6830	1291.6793	2.9
	y_{10}	1180.6508	1180.6473	3.0
	$y_{10}-NH_3$	1163.6220	1163.6208	1.0
	y_8	960.5659	960.5625	3.5
	y_7-NH_3	796.4704	796.4676	3.6
	y_6-NH_3	668.4105	668.4090	2.2
	Y_5	571.3939	571.3926	2.2
BAL: ^{109}ALCSEKLDQWLCEKL^{123}	b_{14}	1761.8345	1761.8299	2.6
	b_{13}	1633.7389	1633.7349	2.5
	b_{11}	1344.6532	1344.6616	−6.3
	y_9	1204.6086	1204.6031	4.6
	y_7-NH_3	959.4655	959.4655	0.0
	b_8	917.4395	917.4397	−0.2
	b_7	802.4096	802.4128	−4.0
BLF: ^{148}FFSASCVPCIDR^{159}	b_{11}	1284.5347	1284.5388	−3.2
	$b_{11}-H_2O$	1266.5295	1266.5282	1.0
	y_{10}	1164.5151	1164.5136	1.2
	$y_{10}-H_2O$	1146.5033	1146.5030	0.3
	b_7-H_2O	781.3341	781.3338	0.4
	y_5	660.3133	660.3134	−0.2

by CIRCA are still largely dictated by the position of the basic residue (and thus there is a bias for y-type cleavage of tryptic peptides), CIRCA avoids the complicated product ion spectra associated with high-energy dissociation methods (e.g., high-energy CID in MALDI-TOF/TOF) and provides tandem mass spectra that can be interpreted almost entirely in terms of b and y fragmentation. Furthermore, this result is accomplished without compromising mass measurement accuracy or mass resolution.

Although there is some low-mass limit to the ability of CIRCA to improve on SORI-CID (presumably due to diminished density of IR-active modes), significant fragmentation information was obtained for singly protonated tryptic peptides ranging from m/z 1300 to m/z 2400, with some information still attainable at m/z 1200. This low-mass cutoff is not a significant limitation, as the tandem mass spectrometry of larger peptide ions is more informative in a proteomic context. The implementation of CIRCA thus

renders MALDI-FTICR-MS/MS suitable for high-accuracy, high-resolution proteomics experiments based on low-energy tandem mass spectrometry of singly protonated tryptic peptides.

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