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Title: 2D FT-ICR MS of Calmodulin: a top-down and bottom-up approach

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Abstract

Two-dimensional Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (2D FT-ICR MS) allows data independent fragmentation of all ions in a sample and correlation of fragment ions to their precursors through the modulation of precursor ion cyclotron radii prior to fragmentation. Previous results show that implementation of 2D FT-ICR MS with IRMPD and ECD has turned this method into a useful analytical tool. In this work, IRMPD tandem mass spectrometry of Calmodulin (CaM) has been performed both in one-dimensional and two-dimensional FT-ICR MS using a top-down and bottom-up approach. 2D IRMPD FT-ICR MS is used to achieve extensive inter-residue bond cleavage and assignment for CaM, using its unique features for fragment identification in a less time- and sample-consuming experiment than doing the same thing using sequential MS/MS experiments.

Introduction

Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR MS) provides the highest resolving power and mass measurement accuracy among all kinds of Mass Spectrometry (MS) [1].
In an FT-ICR MS experiment, the raw signal is measured as a function of time, the time-domain data or “transient”. The Fourier Transform (FT) is then applied, along with a series of operations/algorithms such as zero-filling, convolution [2] and apodisation [3] to improve the quality of data [4], transforming the time-domain data into the frequency domain. A frequency spectrum is obtained and converted to a mass (m/z) spectrum through a calibration function [5]. In order to obtain more structural information, analytes are often isolated and fragmented (tandem Mass Spectrometry, or MS/MS). Tandem MS is influenced by the resolution that can be obtained during isolation and by the various fragmentation techniques, which produce different and often complementary fragmentation [6].

Two-dimensional (2D) FT-ICR MS allows data independent fragmentation of all ions in a sample and correlation of precursor and fragment ions without prior isolation [7–10]. This correlation is obtained through the modulation of precursor ions’ cyclotron radii prior to fragmentation using a series of RF-pulses. Pfandler et al. developed the first pulse sequence used for 2D MS experiments. A different pulse sequence called Stored-Waveform Ion Modulation (SWIM) was developed and applied by Ross et al. [11] and by van der Rest and Marshall [12]. Figure 1 shows the RF-pulses used in a typical 2D FT-ICR MS experiment. The ions enter the cell ideally at the centre, and are then excited by an excitation pulse, P₁. The ions rotate inside the cell according to their cyclotron frequency (ωc) for an encoding time (t₁), to accumulate a phase of ωc·t₁. An encoding pulse (P₂), equal to P₁, is then applied, and the ions are either excited further or de-excited, depending on their instantaneous phase relative to P₂. At the end of P₂, the ions’ cyclotron radii are modulated according to t₁ and ωc [9]. P₂ is followed by a fragmentation period (τₘ), in which the ions are subjected to a radius-dependent fragmentation, and produce fragment ions whose abundances depend on t₁ and the precursor ions’ cyclotron frequency (ωc). After τₘ, P₃ excites both precursors and fragment ions, which can then be detected, as per a normal FT-ICR MS experiment, producing a time domain transient (t₂). A series of transients are recorded at regular increments of t₁, and a two-
dimensional Fourier transform along $t_1$ and $t_2$ is performed: the output is a two-dimensional map showing the correlations between fragments and precursors (Figure 2).

Figure 2 shows the schematic representation of a two-dimensional mass spectrum. The $y$-axis corresponds to the $m/z$ of the precursor ions, while the $x$-axis shows the $m/z$ of the fragment ions. All the signals along the identity line (i.e. the line whose equation is $y = x$) represent the precursor species fragmented during the experiment in correlation with their own cyclotron frequency: for this reason, this is called autocorrelation line. All the signals along a horizontal line constitute the fragmentation patterns of each precursor. All the signals along a vertical line represent the precursor ions of a given fragment ion. Finally, in a 2D FT-ICR mass spectrum it is possible to extract lines parallel to the autocorrelation line, with a difference in intercept corresponding to the mass of a neutral molecule divided by the charge of the precursor ($y = x + m_{neutral}/z$). These are the so called neutral-loss lines, and show the loss of neutrals by precursor ions. Vertical precursor ion spectra and neutral-loss spectra can be easily extracted from two-dimensional maps, constituting information readily available only in 2D MS [13].

2D FT-ICR MS development started in the 1980’s [7,14], but it was hindered by insufficient computational capabilities: the acquisition of an individual transient for each increment of $t_1$ quickly produces large amounts of data that were really challenging for the computers of that time. With recent improvements in computer technology and the improvements in ion fragmentation techniques for FT-ICR MS, an interest to develop 2D FT-ICR MS into an efficient analytical method were renewed [13]. The original 2D MS experiments were conducted using in-cell collisional dissociation [8], with a consistent loss of resolution resulting from fragmentation at a high orbital radius leading to magnetron expansion of the fragment cloud and ion loss. These in cell collisions also required neutral gas pulses and significant pumping delays to return to optimum vacuum pressure prior to detection, slowing limiting analysis. Fragmentation modes which do not involve the use of gases, such as InfraRed Multi-Photon Dissociation (IRMPD) [15] or Electron Capture
Dissociation (ECD) [16] assist in the measurements of high-resolution 2D mass spectra by alleviating some of these limitations [17,18]. An initial disadvantage 2D-FT-ICR MS, which further hindered development, was represented by the number of artefacts which can arise in 2D spectra. These artefacts can come from harmonics (in both dimensions) due to the two-dimensional Fourier Transform [12] or to the fluctuation in the amplitude or frequency of a signal - scintillation noise, the equivalent of t₁ noise in NMR spectroscopy [19]. Different algorithms for the de-noising of 2D mass spectra have been developed [20,21] and are now applied to all contemporary 2D-FT-ICR MS spectra, as a result scintillation noise no longer represents a significant problem to 2D-FT-ICR MS spectra. The potential of 2D FT-ICR MS as an analytical method for the structural analysis of small molecules has been recently demonstrated on cholesterol, showing the possibility for the technique differentiate various fragmentation pathways and interrogate detected species [22]. 2D IRMPD FT-ICR MS has also been used for the analysis of peptides derived from tryptic digestion of commercially available collagen which was not readily achievable with traditional 1D MS/MS [23] and for an ECD/IRMPD comparative study for Proteomics, using digested Cytochrome C as a model [24]. In this work, 2D IRMPD FT-ICR MS was used for the structural analysis of Calmodulin (CaM) [25,26], in both top-down and bottom-up protein tandem mass spectrometry approaches. Calmodulin is a ubiquitous 16k Da protein able to bind up to four Calcium(II) atoms in the human body for signalling purposes. CaM has been used as a standard for deamidation [27] and to investigate the behaviour of anti-cancer drugs at the atomic level [28,29]. Its dimensions and relatively simple structure, given the absence of disulphide bonds, make it the perfect model to perform comparative top-down and bottom-up studies.

**Experimental**
Materials

Bovine calmodulin (CaM), trypsin (TPCK treated from bovine pancreas), and ammonium bicarbonate ((NH₄)HCO₃) were purchased from Sigma Aldrich (Dorset, UK). HPLC grade methanol and formic acid (HAc), were obtained from Fisher Scientific (Loughborough, UK). Water was purified by a Millipore Direct-Q purification system (Merck Millipore, MA, USA).

Sample preparation

Salt adducts were removed from Calmodulin through 3 kDa molecular weight cut-off centrifugal filters (Amicon Ultra 0.5 mL, Merck Millipore Ltd, Tullagreen, Ireland). CaM was then passed through 0.22 µm centrifugal filters to remove particulates prior to nano-electrospray ionisation. For the top-down approach, Calmodulin (7.5 µM) was dissolved in a 75:25 water/methanol (v/v) solution with 0.3% (v/v) of formic acid. For the Bottom-up approach, Calmodulin (aqueous solution, 40 µM) was digested with trypsin in ammonium bicarbonate 100 mM at an enzyme-to-protein ratio of 1:40. A 3kDa centrifugal filter device was used as a reactor at 37 °C, and the digestion was stopped after 4 hours by isolation from trypsin through centrifugation of the peptides through the molecular weight cut-off filter. The tryptic-digest was diluted to an end concentration of ~10 µM using a 75:25 water/methanol (v/v) solution and acidified with 0.3% formic acid (v/v).

Methods

FT-ICR MS was performed on a 12 T Bruker SolariX FT-ICR Mass Spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with a custom nano-electrospray source (nESI). The source used 1.22 mm thin-walled glass capillaries (World Precision Instruments, Hitching, UK) pulled to obtain tips of ~1 µm in diameter with a flaming/brown micropipette puller (Sutter Instrument Co., Novato, California, U.S.A.). The flow rate of the capillaries was ~2 µL/hour in aqueous solution at a
capillary voltage of 1000 V. Each experiment used 10-20 µL of sample. IRMPD fragmentation was achieved using a continuous wave, 25 W, CO₂ laser (Synrad Inc., Washington, USA) held at 70% of its power output. IR photons were produced at a wavelength of 10.6 µm and pulsed into the ICR cell for 0.3 s prior to detection. The pulse program used for the 2D MS analysis was optimised in order to have the highest fragmentation efficiency according to previous studies [30].

For the top-down 2D FT-ICR MS, 512 scans (increments of t₁) of 4M data points were acquired over a mass range of m/z 883.5-3000 m/z on the vertical axis and m/z 147.5-3000 m/z on the horizontal axis, total time of acquisition was ~20 minutes.

For the bottom-up 2D FT-ICR MS, 4096 scans of 512k data points were acquired over a mass range of m/z 368.2-3000 m/z on the vertical axis and m/z 147.5-3000 m/z on the horizontal axis; total time of acquisition was ~50 min. 2D FT-ICR data were processed with a custom program, SPIKE (Illkirch-Graffenstaden, France) [31], which used urQRd as a de-noising algorithm [21]. Data for the 2D MS analysis were processed using a HP desktop computer (Hewlett Packard, Bracknell, UK), via a virtual machine that simulated a Ubuntu 64bit operative system, with 8 GB of dedicated RAM and 4 core processors. For the top-down analysis, a rank of 6 was used for the urQRd denoising algorithm, while this value was equal to 10 for the bottom-up analysis. One-dimensional IRMPD/FT-ICR MS/MS spectra of isolated precursor ions were acquired for comparison to the two-dimensional method. All spectra were internally calibrated using known fragment peaks with a quadratic calibration function, and manually interpreted and assigned. For data interpretation of the one-dimensional mass spectra, Data Analysis v4.1 (Bruker Daltonik GmbH, Bremen, Germany) was used. The parameters used for the one-dimensional and two-dimensional mass spectrometric analysis of CaM are provided in the Supporting Information (table S3).

Results
**Top-down Calmodulin.**

Figure 3 shows the top-down two-dimensional spectrum of Calmodulin (3.a), with the extraction of the autocorrelation line (3.b), a precursor ion (vertical) scan (3.c), and a fragment ion (horizontal) scan (3.d). The 2D mass spectrum in Figure 3.a shows a very clear autocorrelation line, representing the correlation of the precursor ions signal with their own cyclotron radius. Figure 3.b shows the extraction of the autocorrelation line (top) in comparison with a one-dimensional full MS of Calmodulin with the same solution conditions (bottom). CaM shows a wide range of charge states, from 8+ to 17+, as expected under the denaturating conditions used, and that 2D FT-ICR MS is able to detect and fragment them. The signal intensity of the autocorrelation line profile cannot be directly compared with that of the correlating one-dimensional spectrum (Fig 3.b, bottom, signal averaged for 100 scans) because in 2D FT-ICR MS intensities are not only proportional to the abundance of detected ions; they are also dependent on the change in precursor ion intensity during the fragmentation event. Lower intensity sodium/calcium adducted peaks are also visible in Fig. 3b, showing an increase in intensity of the sodium ion-adducted peaks in the 2D FT-ICR MS compared to the 1D FT-ICR MS spectrum. As these two spectra were acquired on different days with different samples, it is important not to over-interpret the variation in sodium-adducted peak intensities, but it could indicate there is a greater relative variation (i.e. fragmentation) of salt-adducted peaks compared to the purely protonated species. Furthermore, because the 2D FT-ICR MS experiment requires a longer timeframe, it is possible that Na⁺ ions leach into solution from the glass capillary, and that would increase the signal of the sodiated species in solution with time. Figure 3.c shows a vertical precursor ion scan for the y₃ ion of CaM, revealing the precursor ions which generate the y₃ ion at m/z 319.198. In 2D FT-ICR MS, such a precursor ion scan is simultaneously detected and available for all fragments. Figure 3.d shows the fragmentation pattern of the 14+ charge state of CaM. It can be seen that the spectrum is very dense, but 2D IRMPD/FT-ICR MS is able to isotopically resolve the fragment ion peaks. The unassigned peaks in spectrum 3.d may derive from secondary fragments and even “sequence scrambled” ions, as is commonly observed in CAD and/or IRMPD
Horizontal profiles have been extracted and analysed for each charge state, giving a cleavage coverage of 23% for the top-down analysis at the specified conditions. Detailed peak assignment tables are included in the Supporting Information (table S1). The dynamic range observed for the reported single scan 2D mass spectrum is ~200. Its value in 1D MS is ~1200, with 200 scans per mass spectrum.

**Bottom-up Calmodulin.**

Figure 4.a shows the two-dimensional IRMPD-FT-ICR MS spectrum of the tryptic –digest peptide mixture from Calmodulin, with the extraction of the autocorrelation line (4.b), a neutral-loss scan (4.c), and a fragment ion (horizontal) scan (4.d). Figure 4.b shows the autocorrelation line profile (top) in comparison with the full one-dimensional (1D) mass spectrum of digested Calmodulin (signal averaged over 100 scans)(bottom). It can be seen from this comparison that 2D FT-ICR MS shows the same fragments present in the digest with few exceptions. A list of the assigned peaks is provided in the Supporting Information for the two-dimensional analysis (Table. S2.1). Figure 4.c shows an example of neutral loss scan, he spectrum shows all the precursors that during fragmentation lose a neutral molecule whose absence would cause a decrease in m/z of 9. According to the neutral loss line equation, \( y = x + m_{\text{neutral}}/z \), for doubly charged ions, this loss would correspond to loss of a water molecule \([M-H_2O]^2+\). Figure 4.c is therefore showing all the doubly charged ions in the mixture which lose a water molecule during IRMPD fragmentation. A neutral-loss line showing water-loss from doubly charger precursors is chosen as an example the capability of 2D-FT-ICR MS techniques: the same type of profile can be applied to other neutral losses, for example phosphate groups or glycans, respectively from phosphopeptides and glycopeptides, and it constitutes information available readily in 2D FT-ICR MS. Figure 4.d shows a fragment ion scan for the ion at m/z 782.380. The horizontal profile (top) is in direct comparison with the corresponding 1D IRMPD FT-ICR MS/MS of the same peptide (bottom). The figures show that results obtained with
2D IRMPD-FT-ICR MS are generally comparable with the one-dimensional approach in terms of fragments shown and sequence coverage, but do not generate the same signal intensities for the different ions. The differences in ion abundances between the shown fragment ion scan and its homologous MS/MS spectrum derive from the different tuning of the two spectra. Even with the same tuning conditions, although, characteristics such as misalignment of the IR laser, the magnetron expansion of the ion cloud and its own expansion due to modulation in 2D MS make that 2D spectra result different in abundances compared to 1D spectra tuned in a similar way. Furthermore, the parameters for 1D MS are chosen in order to obtain an optimal fragmentation of a single precursor ion at a time, assuming that the ions are constantly inside the laser beam before excitation for detection. In 2D MS, the pulse programme is optimised in a way that the ion packets are modulated in order to be fragmented selectively with the variation of $t_i$, causing their oscillation in and out of the laser beam area, and resulting in different fragmentation pattern intensities. The total sequence coverage of CaM from the detected and fragmented peptide obtained with the 2D IRMPD FT-ICR MS of tryptic-digested CaM was 22% (See Supporting Information tables S2.1 to S2.6). The dynamic range observed for the reported single scan 2D mass spectrum is ~1000. The dynamic range observed in 1D MS for a 200 scans spectrum is ~300.

Discussion

2D IRMPD/FT-ICR MS spectra of Calmodulin were acquired using a top-down and a bottom-up approach. The approaches are differ greatly with respect to the type and amount of information they provide, and need to be addressed individually when finding the optimal conditions for acquiring 1D and 2D FT-ICR Mass Spectra.

In every mass spectrometric technique, resolution is influenced among other factors by the number of dataset size during the acquisition. In 2D FT-ICR MS the output is a two-dimensional map with $m$/z along both the axis, as a result it is possible to define the resolution for both dimensions,
vertical and horizontal. Since the y-axis shows the m/z of ions in the mixture prior to fragmentation, i.e. the precursor ions, the **vertical resolution** is analogous to the ability to separate two precursor ion peaks; while since the x-axis shows the m/z of the fragment ions generated by each precursor, the **horizontal resolution** is related to the separation of narrow fragment ion peaks. Due to the nature of the 2D Fourier Transform, the horizontal resolution depends on the number of data points used to acquire $t_2$, while the vertical resolution depends on the number of iterations in $t_1$ (scans). As it can be seen in Figure 1, $t_2$ is the length of the transient acquired after the fragmentation occurs. Thus, $t_2$ is dictated by the number of data points used for each acquisition and the sampling frequency required, and is easily variable, as for all FTMS acquisitions. $t_1$, as described before, is the delay between the identical initial pulses $P_1$ and $P_2$. The starting value of $t_1$ is dictated by the precursor ions of interest, and the number of increments is dictated by the vertical resolution needed to effectively separate those precursors during the subsequent 2D-FT-ICR MS experiment.

In the case of the bottom-up procedure, the tryptic-digest solution presents a complex mixture of peptides, whose precursor m/z peaks must be resolved in order to be able to distinguish different precursor peptides and overlapping distributions (Figure 4.b). The fragmentation of peptides does not generate spectra as dense in peaks as those generated by the fragmentation of whole proteins. As can be seen in Figure 4.c, it is possible to resolve the generated peptide fragment peaks with a relatively low horizontal resolution. However, the increase in sample complexity requires a higher vertical resolution in order to resolve overlapping peaks. In this case, 4096 data points were used for the acquisition in the vertical dimension, 8 times the amount of data points used for the same acquisition in the top-down approach (see below). Since the resolution is proportional to the number of data points used for the acquisition, and that in the vertical dimension this value is determined by the number of $t_1$ time steps, the acquisition of the 2D mass spectrum of digested/complex mixtures would take more time that the top-down experiment due to increased sample complexity. The mass resolving powers for the bottom-up 2D mass spectrum of CaM were $R_{P_Y} = 58766$ and an $R_{P_x} = 1191$ at m/z 400.
The top-down analysis of CaM showed an initial full MS (Fig. 3.b) presenting peaks of different charge states clearly separated in m/z, with no overlapping distributions between charge states. Therefore vertical resolution needed to be able to separate two narrow peaks in this case would be relatively low, especially compared to the bottom-up procedure (above). For this reason only 512 \( t_1 \) increments (time steps/data points) were required to ensure the effective separation of precursors. The contrary applies for the horizontal (MS/MS) scans: after IRMPD fragmentation, the density of the spectrum (Fig. 3.c) increases greatly compared to the peptide spectra above, requiring a higher horizontal resolution to separate different fragments and provide accurate assignments. In this case, 4M data points were used for the acquired transient, corresponding to a horizontal resolution of 422,473 at 400 m/z. Generation of very dense spectra is typical of the top-down approach, which benefits from the resolution and mass accuracy of FT-ICR MS for accurate, resolved assignment. The mass resolving power was measured using the FWHM reported to m/z 400. For the top-down 2D mass spectrum of CaM, the results were: \( \text{RP}_y \) (vertical) of 182 and an \( \text{RP}_x \) (horizontal) of 422473.

Due to the nature of the two-dimensional map produced in 2D FT-ICR MS, it is possible to define a composite resolution, which takes account of the vertical and horizontal dimensions. In effect the "true resolution" of a 2D MS mass spectrum is the product of the resolution in both dimensions, and is approximately equal to the theoretical peak capacity of the experiment. According to this line of reasoning, the 2D mass resolving powers for the previous spectra are the following: \( 182 \times 422473 = 77\text{M} \) for the top-down analysis of CaM and \( 58766 \times 1191 = 70\text{M} \) for the bottom-up procedure, both at 400 m/z.

The results obtained with 2D FT-ICR MS for both the top-down and the bottom-up analysis of Calmodulin are comparable with those obtained with standard one-dimensional FT-ICR MS, with a consistent saving in time and sample consumption. For the bottom-up approach, the 2D FT-ICR MS
experiment was remarkably faster than a comparative analysis in 1D-MS would be; isolating, fragmenting, and detecting each individual precursor observed in the one-dimensional spectrum of the tryptic digest of CaM could require several hours in comparison. One-dimensional top-down and bottom-up studies on Calmodulin using IRMPD as fragmentation techniques were performed for comparison to the 2D IRMPD FT-ICR MS analysis, showing cleavage coverages of the protein up to 36% for the top-down approach, and about 25% for the bottom-up approach, for a total cleavage coverage of 52% (See Supporting Information, Fig.S1). For the 2D FT-ICR MS experiments the total cleavage coverage obtained with both the approaches reaches ~40% comparable to those obtained in 1D FT-ICR MS. Since precursors have different fragmentation efficiencies, the limitation of using a set fragmentation parameter combination for all charge states is non ideal and could account for the differences between the 2D FT-ICR MS data and individually optimised 1D data. Similar issues arise for LC-MS/MS experiments.

The experiments performed in this work constitute the first example of 2D FT-ICR MS for top-down proteomics, and show that top-down analysis can be performed in 2D MS. In this case, CaM is used as a model for its structure and size, and experiments performed with such a protein constitute the initial step for top-down analysis of complex mixtures. In fact, although the sample used for this analysis was relatively pure, once the procedure is developed it is possible to apply top-down 2D MS on non-pure/complex mixture samples. In such samples, depending on the complexity of the mixture, isolating a protein for standard MS/MS analysis might be difficult if not impossible without contamination from ions close in m/z. As mentioned, in traditional MS/MS the quality of the spectra depend on the resolution obtained with quadrupole isolation of single precursor ions. Narrowing the isolation window in order to include a single ion (e.g. a single charge state of a protein of interest) increases the isolation resolution required, but working with narrow isolation windows in a quadrupole (usually less than 5 Da) inevitably brings substantial signal losses that affect negatively the sensitivity of the final MS/MS spectrum. On the other hand 2D FT-ICR MS provides the fragmentation patterns of all the ions in the mixture without the necessity of
quadrupole isolation, preventing the loss of resolution in the precursor dimension even in case of complex mixtures, as it is demonstrated in this paper for the bottom-up procedure. The resolution in the vertical dimension is higher than resolution typically obtainable with quadruple isolation, and in 2D FT-ICR MS, the vertical resolution is primarily limited by the number of \( t_1 \) steps (vertical scans) acquired, and by the computational capacity available.

2D FT-ICR MS presents particular computational challenges. 2D IRMPD FT-ICR MS of CaM resulted in files of 23.5 GB for both the approaches, a challenging file size for processing and denoising with standard desktop computers like the one used for this work. 2D NMR, by comparison, usually works with files \( 10^3 \) times smaller [33]. The choice of denoising rank is roughly proportional to the number of precursors in the spectrum, and the lower the rank the more the spectrum is denoised [21]. Denoising a mass spectrum highly increases the data processing time (from about 4 hours to ~12 h for the showed 2D spectra in the described work station system), but it is essential for effective visualisation and interpretation. 2D FT-ICR MS development has been hindered until recently due to limited computational capabilities, and computer technology still represents a significant limitation for the technique, but one that is changing rapidly. The current computational approach to processing this data involves parallelizing the 2DFT, denoising, and visualization code and porting the data and code up to parallel computing clusters, which will greatly alleviate the current computational bottleneck.

**Conclusions**

2D IRMPD/FT-ICR MS has been used for the top-down and bottom-up analysis of Calmodulin, and fragmentation patterns of CaM have been obtained with both approaches. The results obtained are comparable with one-dimensional FT-ICR MS/MS and the overall procedure is lower time- and sample-consuming than traditional tandem mass spectrometry. In 2D MS more information is available at once through the observation of the neutral-loss lines and the vertical ion scans. In
conclusion, 2D FT-ICR MS can be used for the identification of proteins in complex mixtures, although the technique could be improved for top-down analysis. The biggest limitation is currently represented by computational capacities.

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Figure 1: Typical pulse programme used for 2D FT-ICR MS experiments. In this work, IRMPD has been used for fragmentation during $\tau_m$. 
Figure 2: Interpretation of a 2D mass spectrum. The autocorrelation line ($y = x$) shows the correlation of the precursor ion signals with their own cyclotron radius. Horizontal fragment ion spectra ($y = m_{\text{precursor}}$) show the fragmentation patterns of each precursor ion. Vertical precursor ion spectra ($x = m_{\text{fragment}}$) show the precursor ions of each fragment ion. Neutral-loss lines ($y = x + m_{\text{neutral}}$) show the loss of neutrals by precursor ions.
Figure 3: Top-down analysis of CaM in denaturing conditions. The 2D mass spectrum (a) has been acquired with 512 scans of 4M data points over a mass range of m/z 883.5-3000 on the vertical axis and m/z 147.5-3000 on the horizontal axis. Three ion scans, in different dimensions, are highlighted: b, the autocorrelation line, in comparison with the full one dimensional mass spectrum of CaM at the same conditions; c, a precursor ion (vertical) scan of the ion m/z 319.197597, indicating all the precursors generating the selected ion (information available only in 2D); d, a fragment ion (horizontal) scan of the ion at charge state 14+, in comparison with an IRMPD FT-ICR MS/MS of the isolated ion with the same m/z. The spectrum d is divided in three spaces to show all the possible assignments.
Figure 4: Bottom-up analysis of trypsin-digested CaM. The 2D mass spectrum (a) has been acquired with 4096 scans of 512k data points over a mass range of $m/z$ 368.2-3000 on the vertical axis and $m/z$ 147.5-3000 on the horizontal axis. Three ion scans, in different dimensions, are highlighted: b, the autocorrelation line, in comparison with the full one dimensional mass spectrum of CaM digest at the same conditions; c, a neutral-loss line (information available only in 2D); d, a fragment ion (horizontal) scan of the ion $m/z$ 782.380488 in comparison with an IRMPD/FT-ICR MS/MS of the isolated ion with the same $m/z$. 
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