Axial spatial distribution focusing: improving MALDI-TOF/RTOF mass spectrometric performance for high-energy collision-induced dissociation of biomolecules

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RATIONALITY: For the last two decades, curved field reflectron technology has been used in matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometers, assisting in the generation of post-source-decay (PSD) or collision-induced dissociation (CID) without decelerating precursor ions, producing true high-energy CID spectra. The result was the generation of product ion mass spectra with product ions typical of high-energy (10 keV and beyond) collision processes. The disadvantage of this approach was the lack of resolution in CID spectra resulting from the excess laser energy deposition used to generate those MS/MS spectra. The work presented in this study overcomes this limitation and includes comprehensive examples of high-energy and high-resolution CID MALDI-MS/MS spectra of biomolecules.

METHODS: The devices used in this study are TOF/RTOF instruments equipped with a high-vacuum MALDI ion source. High-resolution and high-energy CID spectra result from the use of axial spatial distribution focusing (ASDF) in combination with curved field reflectron technology.

RESULTS: A CID spectrum of the P14R1 peptide exhibits product ion resolution in excess of 10,000 (FWHM) but at the same time yields typical high-energy product ions such as w- and [y–2]-type ion series. High-energy CID spectra of lipids, exemplified by a glycerophospholipid and triglyceride, demonstrate C–C backbone fragmentation elucidating the presence of a hydroxyl group in addition to double-bond positioning. A complex high mannose carbohydrate (Man)8(GlcNAc)2 was also studied at 20 keV collision energy and revealed further high-energy product ions with very high resolution, allowing unambiguous detection and characterization of cross-ring cleavage-related ions.

CONCLUSIONS: This is the first comprehensive study using a MALDI-TOF/RTOF instrument equipped with a curved field reflectron and an ASDF device prior to the reflectron. © 2015 The Authors. Rapid Communications in Mass Spectrometry published by John Wiley & Sons Ltd.

The matrix-assisted laser desorption/ionization (MALDI) technique is a soft ionization method known to be particularly well suited for the analysis of large and/or fragile molecules.[1] Since its discovery in the late 1980s,[2,3] this technique has been coupled to several mass-analyzing systems[4–7] but has been extensively used in combination with time-of-flight (TOF) analyzers due to the pulsed nature of lasers. MALDI mass spectrometry has been used to analyze a wide range of biomolecules and in particular lipids, peptides and oligosaccharides.[8–10] Valuable information, when dealing with the structural elucidation of these biomolecules, can be obtained through sequencing experiments where precursor ions isolated using a Bradbury-Nielsen[11] ion gate for example, and then fragmented. The spectra resulting from the fragmentation processes have been called tandem (MS/MS), post-source decay (PSD) and laser-induced-dissociation (LID) spectra. Experiments in which the precursor ions undergo collisions with a neutral gas, such as helium or argon, give rise to collision-induced dissociation (CID) spectra. Such PSD/LID and CID MS/MS experiments can be performed in most MALDI-TOF/RTOF mass spectrometers, depending on the instrumental parameters and setup. The fragmentation process in mass spectrometry has been studied extensively[12,13] and these investigations are ongoing. Importantly, the fragmentation of biomolecules in most MALDI instruments is induced mainly by the excess of internal energy of the precursor ions obtained during the desorption/ionization event.[14–16] The excess of internal energy is usually obtained from the use of a UV laser during the initial desorption/ionization steps.[17]

Cotter et al.[18] described a mass spectrometer capable of generating high-energy (HE) CID (20 keV lab collision energy) MALDI-MS/MS spectra. This mass spectrometer was equipped with a high-vacuum ion generation chamber, and a collision cell followed by an ion selector prior to a
When the laser fluence is close to the threshold for MALDI ions, the axial spatial distribution can be very small so that its contribution to the time-of-flight spread is also small and high mass resolution is achieved. However, when the laser fluence is increased, as is the case for MS/MS, it is the authors’ assertion that the size of the initial axial spatial distribution increases to the point where it dominates the spread in the time of flight and results in low mass resolution.

Since the axial spatial distribution cannot be corrected in the ion source, an additional step is required downstream during the flight of the ions that acts on the axial spatial distribution without disturbing the effect of the pulsed extraction on the axial velocity distribution. This is the function of the ASDF cell and its location is determined by three factors. First, the spatial distribution of the ions along the flight path due to the initial axial spatial distribution has to be very much larger than that due to the initial velocity distribution. Secondly, the ASDF must be carried out in the field-free region where the precursor and product ions are still moving together. Finally, because applying an electrostatic field modifies the ion energies, the ASDF should be after all (or the vast majority) of the product ions have been formed, whether by PSD or CID. The location which satisfies these criteria is just in front of the reflectron, as shown schematically in Fig. 1(B).

The ASDF cell, item 7 in the inset of Fig. 1(B), consists of two high transmission grids spaced 12.5 mm apart where a fast high-voltage pulse can be applied to the grid 1 closest to the ion source and the exit grid 2 is at ground potential. Initially, grid 1 is at 0 V until the precursor (and product) ions of interest, already selected by the ion gate, enter the ASDF cell. At that time, the high-voltage pulse is applied to the first grid and an axial electrostatic field is produced. The result is pulse bunching of the ions such that the spatial distribution is focused just after the exit of the cell. Because the axial spatial distribution in the ASDF cell is predominantly due to the initial spatial distribution in the ion source, the initial spatial distribution is focused while the axial velocity distribution is largely unaffected. Typically, the ASDF focal length is 50 to 80 mm depending on the mass/charge values of the product ions. This range is effectively matched to the transfer characteristic of the MALDI tandem instruments where it dominates the spread in the time of flight.

In this paper, the effectiveness of ASDF in combination with the CFR will be demonstrated by the results of fragmentation of a variety of biomolecules by PSD and CID.

**EXPERIMENTAL**

**Description of the MALDI tandem instruments**

Two different instruments were used to record the CID spectra. The first TOF/RTOF device utilized in this work is a MALDI mass spectrometer based on the Axima series instrument equipped with a curved field reflectron (Axima Performance, Shimadzu Kratos Analytical, Manchester, UK). A detailed description of the instrument can be found.
Typically, the instrument is equipped with a nitrogen laser ($\lambda = 337$ nm) generating a 3 ns pulse width at a maximum pulse rate of 50 Hz. Samples are deposited on a stainless steel microscope slide formatted target (FlexiMass, Shimadzu Kratos Analytical) and the MALDI ion source is operated at approximately $10^{-6}$ mbar. Ions are selected for CID using an ion gate consisting of two Bradbury-Nielsen wire gates. To perform high-energy CID, a gas is introduced into a differentially pumped collision chamber (helium is used as the collision gas), where the pressure is one order of magnitude higher (typically around low $10^{-5}$ mbar) and where ions experience 20 keV collisions because no deceleration of the ion beam (20 kV extraction voltage) is performed. High-energy CID spectra at higher resolution were recorded on the MALDI-7090 instrument (Shimadzu Kratos Analytical). A schematic view of the device is presented in Fig. 1(B). The instrument is a MALDI-TOF/RTOF instrument but with several noticeable changes from the one previously described: the analyzer dimensions are different, now with a total flight path of 4.1 m, precursor ions are formed using a Nd:YAG laser ($\lambda = 355$ nm) pulse rate at 2 kHz, and finally an ASDF cell is located just before the reflectron. Product ions can be obtained with a resolution (full width half maximum, FWHM) of up to 10,000. The instrument is equipped with a curved field reflectron, which allows collection of all product ions, including those generated by metastable/post-source decay (PSD) as well as by CID.

Figure 1. (A) Simplified view representing the various ion distributions in a MALDI source of a mass spectrometer. (B) Schematic and simplified view of the MALDI TOF/RTOF instrument equipped with an ASDF cell: 1 XY sample stage. 2 Source and ion optics. 3 Nd:YAG Laser. 4 CID cell. 5 Ion gate. 6 Reflectron detector. 7 ASDF cell. 8 Curved field reflectron. 9 Linear detector. 10 Helium gas supply. The inset represents the mechanisms by which ions are re-focused in the ASDF cell.
Materials

The MALDI matrices, α-cyano-4-hydrocinnamic acid (α-CHCA) and 2,5-dihydroxybenzoic acid (DHB), were purchased from Laser Bio Labs (Sophia-Antipolis Cedex, France). Ammonium dihydrogen phosphate (ADHP), acetonitrile (MeCN), trifluoroacetic acid (TFA) and 2,4,6-trihydroxyacetophenone (THAP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade methanol (MeOH) and sodium chloride (NaCl) were purchased from Merck (Darmstadt, Germany). Ethanol (EtOH) was obtained from Rathburn Chemicals (Walkerburn, UK). The ProteoMass P14R, MALDI-MS standard and the ether glycerophospholipid 1,2-dihexadecylglycerol-phosphatidylcholine, were also acquired from Sigma-Aldrich. Castor bean oil (pharmaceutical grade) containing as its major triglyceride triricinoleylglycerol was obtained from a local Austrian pharmacy store. Both lipid samples were used without further purification. The complex high mannose oligosaccharide (Man)8(GlcNAc)2 was supplied by Ludger (Abingdon, UK).

Sample preparation

The ProteoMass P14R MALDI-MS standard was used as the example for peptide analysis. The sample was diluted in 70:30 (v/v) MeCN/H2O solution containing 0.1% TFA to give a final concentration of 2 pmol/μL and 0.5 μL was applied to the MALDI target. 0.5 μL of α-CHCA (5 mg/mL in 70:30 (v/v) MeCN/H2O solution with 0.1% TFA containing 10 mM ADHP) solution was added to the peptide sample spot. Finally, the sample spot was dried at room temperature.

The lipid sample preparation was performed according to previously published procedures[23,24] by mixing 0.1–1 mg lipid sample/1 mL MeOH with the MALDI-MS matrix solution (1:1, v/v). The matrix solution contained 15 mg THAP, dissolved in 1 mL of MeOH saturated with NaCl. This mixture was sonicated prior to use. For final sample deposition, 0.5 μL of the analyte/matrix mixture was deposited onto the aforementioned MALDI target and dried at room temperature.

The complex high mannose oligosaccharide (Man)8(GlcNAc)2 was diluted in deionized water, to a concentration of 5 pmol/μL and 0.5 μL was applied to the MALDI target. 0.5 μL of α-CHCA (5 mg/mL in 70:30 (v/v) MeCN/H2O solution with 0.1% TFA containing 10 mM ADHP) solution was added to the peptide sample spot. Finally, the sample spot was dried at room temperature.

RESULTS AND DISCUSSION

MS/MS of P14R (peptide)

The pentadecapeptide, P14R1 was used to demonstrate the type of product ion spectra produced via high-energy (HE, 20 keV) CID both without ASDF and with ASDF. The latter spectrum (Fig. 2(B)) shows the effect of ASDF on the improved resolution of the product ions as well as the precursor ions. The side-chain cleavages (w- and [y-2]-type ions) observed in CID spectra from both instruments are indicative of HE-CID.[25]

Figure 2 depicts comparative CID spectra (precursor ion at m/z 1533.86) taken using the MALDI-7090 (with ASDF) and the Axima Performance instrument (without ASDF). Figure 2(A) exhibits a CID spectrum recorded on the Axima Performance (not equipped with an ASDF cell). A y type ion series is observed along with the w-type ion series but with such low resolution that the isotope envelope is hardly visible. Figure 2(B) represents the CID spectrum recorded with an instrument equipped with the ASDF device (MALDI-7090), where product ions are detected with higher resolution (approximately 10,000 FWHM) – for details, see Fig. 2(C)). With ASDF, the resolution is such that individual isotopes are resolved to the baseline because peak-widths are around 0.1 m/z units. The effect of ASDF is a considerable increase in the resolution of all the product ions (y-, w- and a type ion series) but an improved signal-to-noise (S/N) ratio is also achieved (see Table 1 for details regarding the increase in resolution). The higher S/N ratio means in the end also higher sensitivity. The magnified region in Fig. 2(C) confirms that it was possible to resolve the isotopic envelopes of multiple product ions, with peak resolution obtained in the region of 10,000 FWHM. The product ions w12 and w13 can be observed with relatively high intensity compared to the y12 ion. Ions resulting from side-chain cleavages are typically present due to higher energy collision processes, rather than laser-induced dissociation alone (i.e. when no gas is used). This phenomenon has been described elsewhere[26,27] and a comparison of laser-induced dissociation and HE-CID has also been discussed in an earlier publication.[28]

Further experiments were performed to assess the robustness of this resolution enhancement across varying levels of deposited laser energy. The examples in Fig. 2(D) were acquired with increasing laser energy starting at the threshold for desorption/ionization, then 1.5 and 2 times above the threshold. It is reasonable to expect that since the laser energy and, as such, signal intensity increases, peak resolution would decrease.[18,30] However, it can be observed that even when the laser fluence is set to twice that of the threshold for desorption/ionization, monoisotopic resolution is still achieved. This generates improved S/N ratio as well as increased absolute intensity, whilst maintaining resolved isotopic pattern of product ions. An interesting observation could be made when looking at the intensity of the [y12−2]-product ion, which is observed with intensity similar to that of nearby w ions (w12 and w13), is also due to high-energy collision processes. The [y12−2]-ion is particularly interesting in terms of high-energy collision processes and high-resolution CID spectra as such ions could not be seen (or at least resolved properly) in the spectra without ASDF (Fig. 2(A)). The description of such ions dates back to classical tandem mass spectra obtained from four-sector instruments.[29]

MS/MS of lipids

The major component of castor bean oil, triricinoleylglycerol (ricinoleic acid = 12-hydroxyoleic acid), constitutes an ideal compound for testing the fragmentation properties of...
High-energy (20 keV) CID MALDI TOF/RTOF-MS with helium as collision gas. This is due to the high abundance of charge-remote fragmentation that occurs in high-mass regions. From this mixture triglycerides were desorbed and ionized easily, with the major component generating an abundant sodiated species at \( m/z \) 955.76. Almost no in-source fragmentation could be detected under appropriate ion source conditions, i.e. when the deposited laser energy is slightly above the threshold for analyte desorption/ionization. By selecting the aforementioned, dominant precursor ion using a double Bradbury-Nielsen ion gate width of \( \pm 3 m/z \) units on the Axima Performance instrument,[19] almost the entire

| Product ion or class of compound | Resolution of the product ions without ASDF (AXIMA Performance) | Resolution of the product ions with ASDF (MALDI-7090) |
|---------------------------------|---------------------------------------------------------------|-----------------------------------------------------|
| \( m/z \) 1296.7 (w13)          | 1565                                                          | 10054                                               |
| \( m/z \) 1242.7 (y12)          | 983                                                           | 10734                                               |
| \( m/z \) 1199.7 (w12)          | 1252                                                          | 10632                                               |
| Peptide (P14R)                  | Typically 300–1600\(^a\)                                     | Typically 4000–9900\(^a\)                           |
| Lipids                          | Typically 500–1400\(^a\)                                     | Typically 2900–9900\(^a\)                           |

\(^a\)Depending on \( m/z \) range.

Table 1. Comparison of the resolution for selected product ions of P14R peptide and the typical product ion resolution (P14R peptide and lipids) with and without ASDF.
isotope envelope is selected. This yields one product ion, the B-type ion at m/z 657.50, under typical PSD conditions (data not shown). After introducing helium into the differentially pumped collision cell at a cell pressure of roughly $5 \times 10^{-6}$ mbar the precursor ion is attenuated by approximately 70 to 80% yielding abundant and numerous product ions (see Fig. 3) as previously reported.[23,31]

In particular, high-mass charge-remote fragmentation of the fatty acid substituents is detected, thus elucidating the position of the hydroxyl group and of the double bond. This is seen in Fig. 3(A) where a mass difference of $30 \ m/z$ units ($m/z$ 869.67 to 839.62) indicates the position of the hydroxy group and a mass difference of $54 \ m/z$ units ($m/z$ 839.62 to 785.58) indicates the location of the double bond in the fatty

![Figure 3](image_url)

**Figure 3.** Positive ion mode, high-energy CID of the [M+Na]$^+$ ion of triricinoleoylglycerol ($m/z$ 955.76) using the Axima Performance instrument (A–C) and the MALDI-7090 instrument (D–F): (A) full range CID spectrum; (B) m/z region exhibiting the A-type product ion ($m/z$ 729.55, R = 1200 (FWHM)); (C) m/z region exhibiting the [M+Na]$^+$ precursor ion ($m/z$ 955.76, R = 2200 (FWHM)); (D) full range CID spectrum; (E) m/z region exhibiting the A-type product ion ($m/z$ 729.65, R = 9400 (FWHM)); (F) m/z region exhibiting the [M+Na]$^+$ precursor ion ($m/z$ 955.76, R = 8700 (FWHM)).

![Figure 4](image_url)

**Figure 4.** Positive ion mode, high-energy CID MALDI TOF/RTOF-MS of the [M+H]$^+$ ion of ether 1,2-dihexadecylglycerophosphatidylcholine ($m/z$ 706.64) using the Axima Performance instrument (A–C) and the MALDI-7090 instrument (D–F): (A) full range CID spectrum; (B) m/z region exhibiting charge-remote product ions ($m/z$ 550.53, R = 1000 (FWHM)); (C) m/z region exhibiting the [M+H]$^+$ precursor ion ($m/z$ 706.64, R = 1900 (FWHM)); (D) full range CID spectrum; (E) m/z region exhibiting charge-remote product ions ($m/z$ 550.54, R = 7500 (FWHM)); (F) m/z region exhibiting the [M+H]$^+$ precursor ion ($m/z$ 955.76, R = 14200 (FWHM)).
Figure 5. (A) Positive ion mode MALDI mass spectrum of (Man)$_8$(GlcNac)$_2$ detected as a sodiated adduct ion at m/z 1743.54. (B) m/z region (1100–1400) of the MALDI-PSD-MS/MS (bottom) vs MALDI-CID-MS/MS (top) mass spectra where cross-ring cleavages (X$_3$) are clearly visible. (C) m/z region (1400–1500) with PSD-MS/MS (bottom) and CID-MS/MS (top) spectra exhibiting an X$_4$ cross-ring cleavage.
acid alkyl chain. Additional product ions include the loss of RCOOH (B-type ion, \(m/z\) 657.50) and the structurally diagnostic \(E_{1/3}^{}\) (\(m/z\) 433.27), \(F_{1/3}^{}\) (\(m/z\) 419.26), \(G_{1/3}^{}\) (\(m/z\) 437.25) and \(J_{2}^{}\)-type ions (\(m/z\) 437.25). The latter two structurally diagnostic product ions are typically only observed under very high-energy (20 keV) CID conditions.

When using the Axima Performance, the precursor ion resolution is between 2200 and 2900 (FWHM), with product ions typically at 500–1400 resolution depending on the \(m/z\) range (see Figs. 3(B) and 3(C)). When selecting the same sodiated triacylglycerol precursor ion as measured with the Axima Performance and fragmenting on the MALDI-7090 (with ASDF applied), a significant improvement in resolution of the precursor ion is observed (see Fig. 3(F)) \(R = 9000–12500\) (FWHM). In addition to this, a dramatic increase in resolution of product ions (\(R = 2900–9900\) (FWHM)) depending on the \(m/z\) range of the product ions is obtained. The overall fragmentation patterns for both tandem TOF instruments appear to be very similar with only minor relative intensity variations. A summary of those results with and without ASDF can be found in Table 1.

Another example demonstrating the capabilities of 20 keV collisions with the ASDF implementation for the structural elucidation of lipids is the analysis of the ether glycerophospholipid 1,2-dihexadecylglycerophosphatidylcholine. This lipid easily desorbs/ionizes as a protonated molecule with high abundance and without significant in-source fragmentation.

High-energy CID of this precursor ion (\(m/z\) 706.64) shows significant high-mass charge-remote fragmentation with a mass differences of 14 \(m/z\) units for product ions originating from the two hexadecyl alkyl chains (see Figs. 4(A) and 4(D)). The highly abundant low-mass ions are characteristic for the phosphatidylcholine polar head group with the diagnostic ions \(^{13}C_{3}^{}\) (\(m/z\) 226), \(^{13}C_{2}G_{4}^{}\) (\(m/z\) 184), \(^{13}C_{2}G_{5}^{}\) (\(m/z\) 166), \(m/z\) 104 (choline) and \(m/z\) 86 (dehydrated choline). The product ion nomenclature used for this analyte group is according to Pittenauer and Allmaier.[24]

Based on these initial comparative measurements on two different types of glycerolipids, the fragmentation patterns obtained with the two instruments are readily comparable. The increase in mass spectrometric resolution for precursor ions is approximately a factor of 4–7.5 (see Figs. 3(C), 3(F), 4(C) and 4(F)) and approximately 7.5 for product ions (see Figs. 3(B), 3(E), 4(B) and 4(E)). When using the ASDF device (in the MALDI-7090) the identification of neighbouring product ions separated by 1 \(m/z\) unit becomes much easier and as such allows for the straightforward structure-related interpretation of lipid CID spectra in lipidomic projects.

### MS/MS of oligosaccharide

The spectrum shown in Fig. 5(A) is a MALDI mass spectrum of the Man₈(GlcNac)₂ oligosaccharide recorded using the DHB matrix in positive ion mode and detected as a sodiated species [M + Na]⁺. Figures 5(B) and 5(C) represent selected \(m/z\) ranges of the MALDI-CID and -PSD spectra of the Man₈(GlcNac)₂ sodiated species. Oligosaccharides are usually observed using DHB matrix and are frequently detected as sodiated species.[10] The CID spectrum of this compound has also been recorded on a MALDI-TOF/RTOF instrument but without an ASDF cell.[19] In this latter study, it was found that the CID spectra of Man₈(GlcNac)₂Na exhibit B- and Y-type ions[32] mainly when the low-energy collision regime conditions are met. Those types of product ions provide information related to the interglycosidic linkage of the carbohydrate chain. However, in high-energy collision processes, product ions related to cross-ring cleavages lead to more detailed structural information concerning the carbohydrate branching and modifications.[10] These X-type ions are usually obtained with lower resolution in MALDI-derived CID spectra. This is because the user generally needs to increase the deposited laser energy in order to obtain these product ions. This is especially true with the DHB matrix, which is known to be a so-called ‘cold’ matrix.[33]

In Fig. 5(B), it is clearly visible that the product ions \(^{15}X_{30}^{}\) and \(^{13}X_{3a}^{}\) from the CID spectra of the sodiated Man₈(GlcNac)₂ molecule, recorded with the MALDI-7090 and ASFD on, are obtained with monoisotopic resolution (spectra on the top). An interesting observation is the relatively lower intensity of this species when recording the PSD spectra using exactly the same conditions (laser fluence, matrix, etc.). The only difference in the CID spectra from the PSD spectrum is the use of helium gas in the differentially pumped collision cell. The same observation is made when looking at another \(m/z\) range (Fig. 5(C)). In this latter case the \(^{15}X_{4a}^{}\) product ion appeared to be much more pronounced in the CID spectrum and is almost not visible in the PSD spectrum. It is important to note that our observation is also valid when we compare the relative abundance of the product ions with each other.

### CONCLUSIONS

This paper describes the generation of MALDI-TOF/RTOF spectra with high resolution at “true” high energy using a curved field reflector in combination with ASDF. CID spectra from a wide range of biomolecules are shown. The CID spectra of two lipids, triricinoleoylglycerol and 1,2-dihexadecylglycerophosphatidylcholine, served to demonstrate that it was possible to generate high-energy and high-resolution tandem TOF spectra. In turn, this allowed the detection of C-C backbone fragmentation processes as well as the location of the hydroxyl group and double bond within the fatty acid moiety with high confidence. The example of the P₁R₁ peptide demonstrates the dramatic effect of ASDF in terms of resolution enhancement (in excess of 10,000 (FWHM)) but also the improved signal-to-noise ratio, i.e. tandem spectra containing isotopically resolved product ions with improved sensitivity. The carbohydrate (Man₈(GlcNac)₂) was chosen for the comparison of CID and PSD spectra when using ASDF. The CID spectra exhibited not only linkage-related product ions (Y- and B-type ions), but also cross-ring cleavage product ions (X-type ions).

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