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- 2D FT-ICR MS correlates precursor and fragment ions for all compounds in a complex sample without precursor ion isolation.
- We recorded the 2D mass spectrum of cholesterol using Atmospheric Pressure Photolysis (APPI) for the first time.
- We recorded the positive mode nanoESI 2D mass spectra of a tryptic digest of cytochrome C using both ECD and IRMPD as fragmentation modes.
- We discuss the advantages of 2D FT-ICR MS over MS/MS and LC-MS in terms of the information available in 2D mass spectra.

pulse sequence of this experiment is shown in Fig. 1 [1-9].

- Transients are recorded with regularly incremented values of t_1 . A double Fourier transform according to t_1 and t_2 shows correlations between precursors and fragments in a two-dimensional map.

- **The autocorrelation line** ($y = x$) shows the correlation of the precursor ion signal 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.
- **Electron capture lines** ($y = (n-p) \cdot x/n$) show the capture of p electrons by n -charged precursor ions.
- **Neutral loss lines** ($y = x + m_{\text{neutral}}$) show the loss of neutrals by precursor ions.



Figure 1 is a mass spectrum plot showing the precursor ion (M^+) versus the fragment ion (m/z). The x-axis is labeled "Fragments" and ranges from 212 to 220. The y-axis is labeled "Precursors" and ranges from 365 to 390. The plot shows several peaks corresponding to different fragment ions, with their chemical formulas labeled above them. The peaks are as follows:

- $(M^+ - H_2O - C_{11}H_{23})^+$ at $m/z \approx 387$
- $(MH_2O - C_{11}H_{22})^+$ at $m/z \approx 370$
- $(MH_2O - C_{11}H_{21})^+$ at $m/z \approx 368$
- $(MH_2O - C_{11}H_{20})^+$ at $m/z \approx 366$
- $(MH_2O - C_{11}H_{19})^+$ at $m/z \approx 364$
- $(MH_2O - C_{11}H_{18})^+$ at $m/z \approx 362$
- $(MH_2O - C_{11}H_{17})^+$ at $m/z \approx 360$
- $(MH_2O - C_{11}H_{16})^+$ at $m/z \approx 358$

The plot also includes a dashed red line at $m/z \approx 387$ and a solid red line at $m/z \approx 368$. An arrow points to the peak at $m/z \approx 387$.

Figure 4: APPI 2D IRMPD FT-ICR mass spectrum of cholesterol (zoom).



- Cholesterol sample at 100 pmol/μL in acetonitrile/water (75:25).
- 12 T Solarix FT-ICR mass spectrometer (Bruker) equipped with a Bruker II APPI source.
- 2048 scans of 128k datapoints were recorded over a m/z 36.9-500 horizontal and m/z 184.27-500 vertical mass range.
- IRMPD: Synrad CO₂ laser (25 W), 10.6 μm wavelength, 0.1 s irradiation at 50% power.
- Data processing: NPK (NMR Processing Kernel), rewritten in 64-bit Python programming language. Processed datafiles in HDF5 file format.

- APPI is a continuous ion source that generates both protonated and radical ion species. In the case of cholesterol, in-source ion loss of H_2O and CH_3^{\bullet} are also observed.
- In the context of 2D FT-ICR MS, all fragments generated in the front-end of the mass spectrometer are considered as precursors.
- The 2D mass spectrum shows the fragmentation patterns of all precursors (sample, contaminants and in-source fragments).
- The number of datapoints in the vertical (precursor ion) dimension lead to a 1 Da separation for precursor m/z ratios: we can see the difference between the fragmentation pattern of the protonated ion and the radical ion.
- The vertical precursor ion scans enable the identification of the precursors of each fragment ion generated during the fragmentation period of the 2D FT-ICR pulse sequence.
- Vertical precursor scans lead us to information on the fragmentation mechanism of cholesterol: loss of CH_3^{\bullet} can happen before or after loss of H_2O . (figure 5)

- Tryptic digest of cytochrome C purchased from Thermo Scientific at 800 fmol/μL in acetonitrile/water (25:75).
- Positive mode nanoESI on 12 T Solarix FT-ICR mass spectrometer (Bruker).
- 2048 scans of 128k datapoints were recorded over a m/z 147.4-3000 horizontal and m/z 147.4-3000 vertical mass range for both 2D mass spectra.
- IRMPD: 0.2 s irradiation at 50% power. ECD: 0.05 s irradiation.
- Data processing: NPK (NMR Processing Kernel), rewritten in 64-bit Python programming language . Processed datafiles in HDF5 file format.



Table 1: Sequence coverage of the peptides from cytochrome C using both 2D mass spectra (black: peptides identified in the chromatogram, red: peptides identified in the high resolution FT-ICR mass spectrum).



- 2D FT-ICR mass spectrum of a tryptic digest of cytochrome C with ECD and with IRMPD (40 minutes of analysis time in total).
- We obtain the fragmentation patterns of ion species in a range of abundances, for various charge states. Because 2D FT-ICR MS is data-independent, we observe the fragmentation patterns of ion species that are not listed in the chromatogram provided by the supplier.
- We observe charge reduction lines in the 2D ECD mass spectrum (Figure 7).
- We observe neutral loss lines that are parallel to the autocorrelation line in the 2D IRMPD mass spectrum (Figure 8).
- Sequence coverage of cytochrome C using both 2D ECD and IRMPD mass spectra after tryptic digest: 66%

- TGPLNLHGLGR³⁺ at *m/z* 390.2122 and MIFAGIK²⁺ at *m/z* 390.2278 require a separation power of $m/\Delta m = 25000$ in MS/MS correlate fragments and their precursors in MS/MS.
- Fragmentation: ion of mass *m* and charge *z* loses *p* charges and *n* mass:

• The isotopic distribution of the fragment yields (z-p), and the slope along the peaks of the isotopic distribution of the fragments yields z: this enables the attribution of the precursor for each fragment.

- We have expanded the capabilities of 2D FT-ICR MS to continuous ion sources other than EI and nanoESI by using APPI as an ion source.
- 20 minute-long experiments lead to precursor ion separation of 1 Da, which enables the analysis of increasingly complex samples by 2D FT-ICR MS, as well as the study of the different fragmentation patterns of protonated and radical ions.
- 2D FT-ICR MS allows for the differentiation of fragments generated in the front-end of the instrument (on autocorrelation line) and fragments generated in the ICR cell (fragment peak), which gives us more accurate information on fragmentation mechanisms.
- The slope of fragment isotopic distributions allows for correct precursor identification when they are close in m/z ratio but with different charge states.

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