Creating unique fragmentation in a flash: Ultraviolet Photodissociation (UVPD) isn’t just for large molecules

When traditional fragmentation methods don’t lead to unambiguous characterization of compounds, how can UVPD help?

When using mass spectrometry (MS), and in-particular high-resolution accurate-mass (HRAM) MS for structural elucidation of small or large molecules, scientists normally consider the use of well-established fragmentation techniques such as Collision Induced Dissociation (CID), Higher Energy Collisional Dissociation (HCD), or more recently Electron Transfer Dissociation (ETD). Each fragmentation technique is well characterized for large- or small-molecule applications and are supported by existing software for predictive fragmentation routines or reference mass spectral libraries to evaluate experimental data. In addition, each dissociation method can generate slightly different product ions and/or distribution profiles which can provide complementary information.

The analysis of complex biotherapeutics using top- and middle-down techniques has grown due to the ability to rapidly verify primary sequence, or to identify the site of post-translational modifications which could be lost when performing characterization using a bottom-up approach. However, a limitation of top- and middle-down characterization is that, even when combining the sequence coverage obtained using ETD and a hybrid fragmentation approach called EThcD (a combination of ETD and HCD), you may only be able to account for 50–60% of the sequence.

Adding an orthogonal fragmentation technique, UVPD allows for improved characterization of biotherapeutics through the generation of backbone fragments which can complement those obtained when using ETD and EThcD raising sequence coverage to 70–90%.
In comparison, the structural determination of small molecules using CID or HCD can be challenging due to significant chemical diversity, structural diversity, limited mass spectral library reference coverage, and labile bonds which can result in cleavage of certain important groups (e.g., flavonoid glycoside conjugates, glucuronides) resulting in the reduction or absence of diagnostic fragment ions.

**What is UVPD?**

UVPD differs from traditional fragmentation techniques such as CID and HCD, in that it utilizes photons (~6 eV per photon) generated from a UV laser source to increase the internal energy of a selected precursor ion (electronic excitation), until there is sufficient internal energy present to overcome the barrier for dissociation, therefore generating fragments.

The commercial laser produces energetic photons with a wavelength (213 nm) that overlaps with absorbance bands for several common bond types such C=C (alkenes) and C=N (imines). This provides an advantage compared to CID and HCD where typically a limited number of fragments are generated when there are fused rings present. Therefore, absorption of only a single photon, which occurs on the microsecond timescale, is required for unimolecular dissociation. Typical pulse durations can be 50–100 milliseconds due to much smaller ion beam in the linear ion trap relative to the laser beam. Despite the pulse duration being longer than CID or HCD, UVPD is still compatible with a chromatographic timescale unlike other fragmentation modes such as Electron Induced Dissociation (EID), which generate similar fragmentation patterns.¹

---

**Collision-Induced Dissociation (CID)**

A method of collision-induced dissociation that occurs in the high pressure cell in the linear ion trap. The precursor ion’s kinetic energy is increased by applying an on-resonance frequency and then allowed to collide with helium which converts kinetic to internal energy resulting in fragmentation. Due to the low molecular weight of helium, collisional activation results in lower internal energy preferentially dissociating into low-energy product ions.

**Higher Energy Collisional Dissociation (HCD)**

A method of collision-induced dissociation that occurs in the ion routing multipole (IRM), shown in Figure 1. The IRM consists of a straight multipole mounted inside a collision-gas filled tube. A voltage offset between the C-trap and IRM accelerates parent ions into the collision gas inside the IRM, which causes the ions to fragment into product ions. The product ions are then sent to the ion trap or the Thermo Scientific™ Orbitrap™ mass analyzer for mass analysis. HCD produces triple quadrupole-like product ion mass spectra.

---

Figure 1. Schematic representation of a Thermo Scientific™ Orbitrap™ Tribrid™ mass spectrometer highlighting the location of the UVPD source, along with other key components.
How can UVPD help with small-molecule characterization?

For many small-molecule compounds, CID and HCD can provide sufficient fragmentation to aid in characterization, and with the ever-increasing number of compounds mass spectral libraries such as mzCloud, there is a greater chance of a match.

However, some compounds pose significant challenges when it comes to structural characterization. These could be identification of structural isomers, site of glucuronidation, and double bond formation within acyl chains of lipids, or the characterization of aromatic compounds as shown in Figure 2.

Being able to utilize an alternative fragmentation technique which provides different mechanism of activation and an increase in fragmentation energy can, depending upon a compound’s structure, provide increased and unique diagnostic fragmentation to solve some of the challenges mentioned earlier.

Electrospray ionization (ESI) generates Even Electron (EE) species, where the [M + H]+ species is the predominant precursor ion selected for fragmentation. CID typically only generates low-energy fragment ions, which may be limited. The higher collision energies involved with HCD can induce EE product ions as well as OE fragments, potentially extending structural coverage. With its increased energy deposition across pi bonds, UVPD readily generates a mixture of EE and OE fragments that can significantly increase the formation of structurally significant product ions.

The data presented within this Smart Note were analyzed using the extensive capabilities of Thermo Scientific™ Mass Frontier™ software which makes analyzing fragmentation data from any dissociation technique simple to perform, and most importantly scientifically relevant, when providing fragmentation proposals. With more than 52,000 fragmentation schemes, 217,000 individual reactions, 256,000 chemical structures and 216,000 decoded mechanisms from peer reviewed literature, the software uses real data rather than in silico predictions which are limited to what was coded.

Figure 2. A comparison of using HCD to fragment 4’-hydroxy diclofenac (Top) and 5-hydroxy diclofenac (Bottom) where the monoisotopic precursor ion of m/z 312.02 was selected. There are significant diagnostic ions present in either of the two spectra, demonstrating that HCD fragmentation was unable to confidently differentiate between the two structural isomers.
As shown in Figure 3, the UVPD fragmentation spectra for the structural isomers of hydroxy diclofenac exhibit both increased fragmentation and the presence of diagnostic ions, allowing confident determination of each isomer; a result not possible using traditional HCD.

UVPD can also be useful in determining the site of metabolite conjugations. Glucuronidation can occur at different sites on a target compound with no net change in molecular weight, such that the isomers must be chromatographically separated and diagnostic fragmentation available to permit confident identification of the modification site. Darunivir can have an O- or N-linked glucuronide conjugate, as shown in Figure 4.

Figure 3. UVPD fragmentation spectra for (a) 4'-hydroxy diclofenac and (b) 5-hydroxy diclofenac acquired using a 65 ms pulse duration, highlighting the increased amounts of fragmentation observed when compared to the HCD fragmentation shown in Figure 2. The fragmentation highlighted by the red boxes is expanded (c, d) to show the complementary fragmentation for the 4'- and 5-hydroxy diclofenac, and the presence of a diagnostic fragment for 5-hydroxy diclofenac (d). The fragments highlighted by red text also show diagnostic fragmentation.

Figure 4. The compound Darunivir (center) can be metabolized at different sites, so understanding where the site of metabolism occurs is important. UVPD fragmentation can help scientists to elucidate between O- and N1- glucuronides.
When comparing HCD and UVPD fragmentation for O-Glu Darunivir (data not shown), HCD produced five primary fragments, whereas UVPD produced twice as many. For confident characterization of the site of metabolism, HCD produced two supporting fragments, whereas UVPD produced eight which leaves no doubt as to the site of glucuronidation. Figure 5 shows extensive UVPD fragmentation for both N1- and O-glucuronidation of Darunivir, demonstrating that, for certain compounds where there is little to no fragmentation generated by CID or HCD, UVPD can provide the fragmentation information needed confirm or deny structural assignments.

**Summary**

While traditional fragmentation techniques such as CID, HCD, and the more recent ETD and EThcD provide sufficient fragments to aid in structural diagnostics, this is not always the case. Here, UVPD can be a useful fragmentation method for compounds containing C=C (alkenes) and C=N (imines) bonds. As such, UVPD can be advantageous for the analysis of a wide range of small-molecule compounds such as flavonoids, fatty acids, and phospholipids.²

The UVPD fragmentation technique offers several advantages:
- More informative fragmentation for a wide variety of compounds
- Greater sequence coverage for intact proteins and unique structural information for small molecules
- Unlike ETD fragmentation, not limited by the precursor charge state
- Increased simplicity because the only parameter used is activation time, which is determined and set automatically during method runs based on precursor molecular weight and charge state

Orbitrap Tribrid Mass Spectrometers offer a truly unique combination of fragmentation techniques, and the ability to perform high-resolution MSⁿ fragmentation with easy to use template-driven method setup, making the insights in your data more easily accessed.
References