The "ETD-like" Fragmentation of Small Molecules

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Poster Note 64772

ABSTRACT

Purpose: To investigate the potential use of ultraviolet photodissociation (UVPD) to provide unique and potentially diagnostic fragmentation information for structure determination of small molecules, specifically flavonoids and related compounds

Methods: Fragmentation data was acquired on several pure standards of compounds submitted to fragmentation by resonance excitation trap collision induced dissociation (CID), high energy collisional dissociation (HCD), and UVPD.

Results: Laser-induced fragmentation provided unique fragments or enhanced the detection of kinetically unfavorable fragment ions in many of the compounds analyzed. These unique fragments provide additional information on the compounds studied, which could be used to infer structure information.

INTRODUCTION

The determination of structure for unknown small molecules is a particularly difficult challenge. Typically, fragmentation data is acquired and used to compare against a reference spectral library or provide information about potential structure components due to historical knowledge of common fragmentation behavior. However, when the reference libraries do not contain the molecule in question, similarity searches can provide an indication of possible substructure or structure class. These approaches are limited when considering compounds like flavonoid glycoside conjugates where the nature and positional linkage of the glycoside can be difficult to determine due to the labile nature of glycosidic bond under collision induced fragmentation. Ultraviolet photodissociation, in which a laser is used to provide the energy to a trapped molecular ion to drive fragmentation, has been applied in many proteomic applications to provide diagnostic information[1,2]. Here, we apply UVPD to several small molecules to determine its utility for their structural determination.

MATERIALS AND METHODS

Sample Preparation

Standard material for several flavanoids and their conjugates including flavone, flavanone, quercetin, quercetrin, quercetin-3-rutinoside, quercetin-3-xyloside, rhoifolin, robinin, prunin, luteolin, homorientin, luteolin-7-O-glucoside, and luteolin-4'-O-glucoside were provided by Dr. Mark Berhow. In addition, standards for orientin, luteolin-3',7-O-glucoside, and luteolin-3'-glucoside were obtained from IndoFine Chemical Company (Hillsborough, NJ). Standards of theobromine, caffeine, hypoxanthine, and 7-(2,3-dihydroxypropyl)theophylline (DHP-theophylline) were obtained from Fisher Scientific. Standards were prepared to concentrations of between 0.5 and 2 µg/mL in 1:1 MeOH:1mM ammonium acetate in water.

Mass Spectrometer Acquisition Conditions

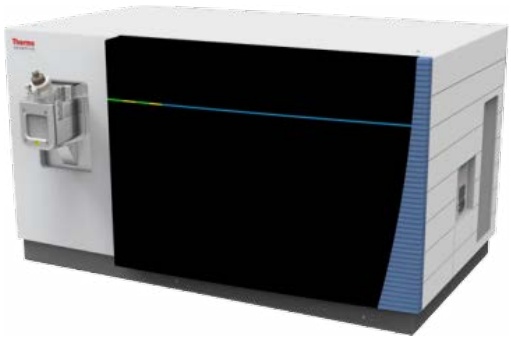
Mass spectrometer: Thermo Scientific™ Orbitrap Fusion™ Lumos™ Mass Spectrometer

Laser: 193nM 200Hz at 1.5mJ/pulse 30 pulses or 3 mJ/pulse 10 pulses
213nM 2.5 kHz at 2.5 µJ/pulse, between 60-800 pulses

Laser energy (for 193 nM laser) and pulse rate (both wavelengths) were optimized for each compound.

Compounds were infused into the mass spectrometer under positive mode ESI and fragmentation was obtained using CID, HCD, and UVPD. In each case, the energy or pulse rate was optimized to provide the best fragmentation information. For CID and HCD this was performed by adjusting the collision energy for maximum fragmentation of the parent molecular ion while retaining the best possible fragment spectra complexity. UVPD energy or pulse count was optimized to provide the best signal for fragmentation in cases where near-complete fragmentation of the parent molecular ion could not be achieved.

Figure 1. Front view of the Orbitrap Fusion Lumos Mass Spectrometer.



UVPD Lasers

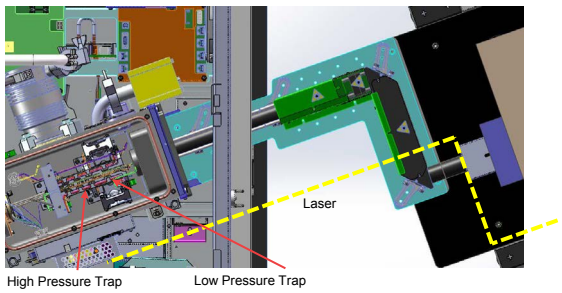
For this work, we investigated two different lasers, which provided different wavelengths.

The first was an excimer laser with a wavelength of 193nm. An excimer (excited dimer) laser functions by generating a high energy reaction between noble gas molecules or a noble and a reactive gas (exciplex – excited complex). The bound high energy state pseudo-molecules undergo emission to a repulsive ground state where they dissociate. During this process, laser light in the UV range is generated.

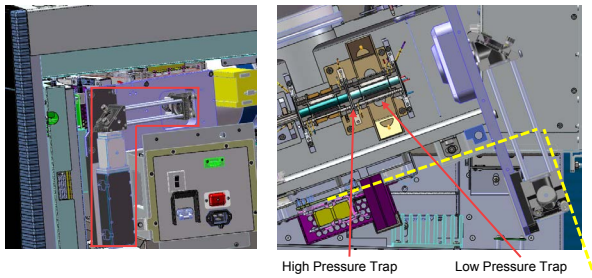
The second laser was a Nd:YAG (neodymium doped yttrium aluminum garnet) laser, which is an optically pumped laser. Typically, these lasers emit in the infrared range (>1000nm), however, when operated in a pulsed Q-switching mode, where the laser energy is released in a pulse when reaching a threshold. Frequency doubling of the pulses can be used to obtain shorter wavelengths and in the laser used in this study, the fifth harmonic was used with a resulting 213nm.

The lasers were mounted such that the path entered the dual linear ion trap with fragmentation occurring in the high pressure region (Figures 1 and 2). Each laser also provided a degree of freedom to adjust the total energy imparted to the study molecules through adjusting the pulse rate and/or pulse energy.

Figure 2. Orientation of the lasers onto the Orbitrap Fusion Lumos Mass Spectrometer.



Note: Top view of excimer laser path through linear ion trap low and high pressure cells.



Left: Exterior view of the right side of the mass spectrometer showing mounting of the Nd:YAG laser. Right: Top view of the laser path through the linear ion trap low and high pressure cells.

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Ultraviolet Photo Dissociation

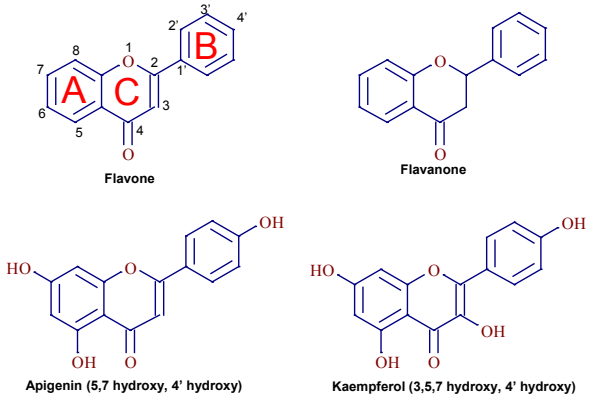
Photodissociation utilizes the energy of incident photons to increase the internal energy of a target molecule until it has sufficient energy to overcome its barrier for dissociation. As opposed to collisional induced fragmentation mechanisms, where energy is imparted through collisions of the molecular ions with gas molecules, the photons in UVPD are directly absorbed by target molecules depending on their UV absorption profile. Typically, a single photon does not impart sufficient energy for fragmentation and the absorption of multiple photons is required to reach a sufficiently excited state. This is why UVPD is typically also referred to as infrared multiphoton dissociation (IRMPD). It is important to consider the number of photons required for a specific structure class of molecules and the time over which those photons are delivered, since the input of energy is balanced against the internal redistribution of vibrational energy when determining the kinetically most favorable fragmentation pathways observed.

RESULTS

Compound Structure and UV Absorption

We studied the UVPD of two major categories of compounds—flavonoids and xanthines. Flavonoids are a broad class of plant secondary metabolites, which generally have a 3-ring structure comprised of two phenyl rings (A and B) and one heterocyclic ring (C) typically linked with A. Two examples are shown in Figure 2 for flavone and flavanone. Different flavonoids posses different hydroxy functions in either the 3,5,6,and 7 position on the AC rings or the 2',3', and 4' position on the B ring. Examples of core structures apigenin (aglycoside of rhoifolin) and kaempferol (aglycoside of robinin) are also shown in Figure 3.

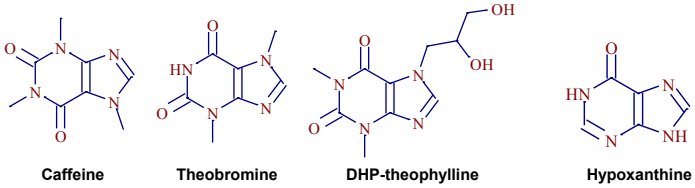
Figure 3. General Flavonoid CoreStructures and Example Flavonoids.



Glycosides of apigenin, kaempferol, and luteolin were studied for potential unique fragmentation

An additional class of compounds studied were around the xanthine core structure (Figure 4). These molecules all have a maximal UV absorbance at low wavelengths (200–220 nm) with a secondary absorbance around 265–280nm, with the exception of hypoxanthine, where the absence of the second carbonyl results in a lower absorbance in the 200–220nm range and a shift in the second absorbance peak to lower wavelengths (250nm). The wavelength of the Nd:YAG laser used for these compounds, 213nm, corresponds better to the absorbance of theobromine, caffeine, and DHP-theophylline.

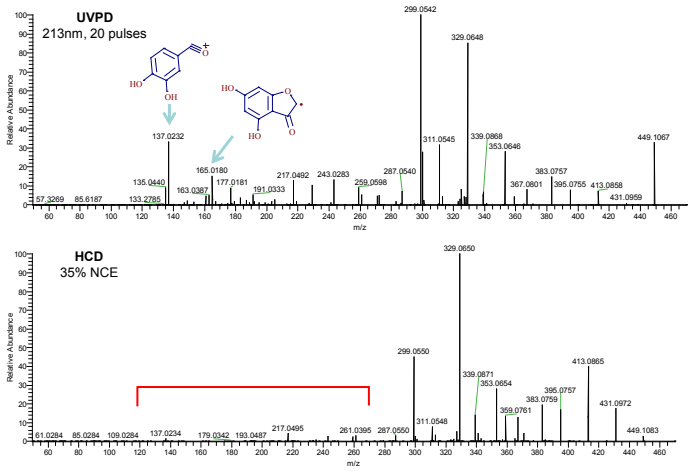
Figure 4. Xanthine Compounds.



Flavonoid Conjugate Behavior

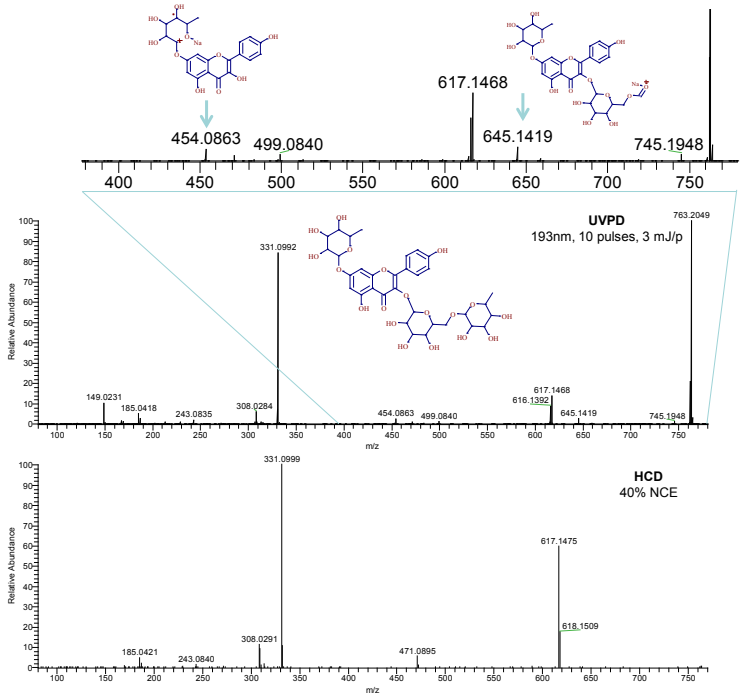
We investigated the fragmentation behavior of several flavonoids and their conjugates to determine diagnostic fragmentation. When submitted to UVPD (213 nm), flavone and flavanone both produce unique fragment ions from radical fragmentation channels not observed in either CID or HCD (not shown). This led to the hypothesis that unique fragmentation could be obtained for structure determination. A study of luteolin and several of its monoglycosides showed that fragmentation of the core structure could be obtained concurrently with the losses of the glycosides typically observed by other fragmentation techniques (Figure 5). This indicates that the kinetics of fragmentation for UVPD is different from those of collisional induced fragmentation and supports the hypothesis that, with the right wavelength/photon energy, we could obtain positional information from fragmentation data.

Figure 5. Fragmentation of Luteolin 8-C-glycoside (Orientin) by UVPD.



Utilizing the higher energy output of the excimer laser (193nm, 3mJ/pulse) and looking at complex 3-glycoside conjugate compounds, like robinin and rhoifolin, UVPD created unique fragmentation from the glycosides *in situ,* which could prove useful for identifying the nature and linking order of the glycosides (Figure 6). Subsequent studies will be required to further optimize the wavelength and energy sufficiently to activate these pathways.

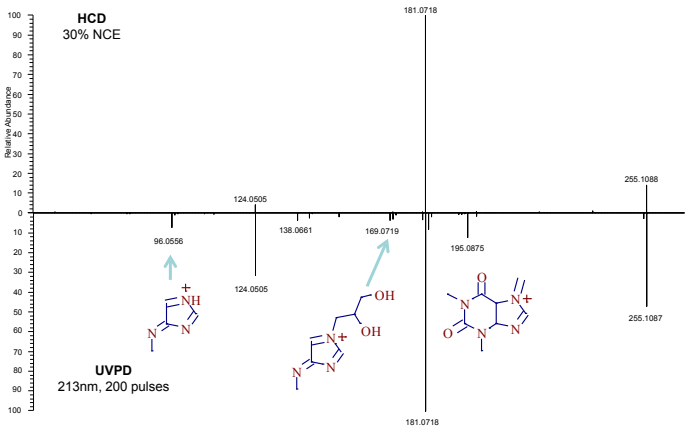
Figure 6. Fragmentation of Robinin by UVPD (193nm, 3mJ/pulse) and HCD (30% NCE).



Xanthine UVPD Fragmentation

For all molecules, we compared the UVPD fragmentation to HCD as for flavonoids. Consideration was also taken for the pulse count required to provide fragmentation by UVPD. For DHP-theopylline, theobromine, and caffeine, pulse counts of between 200 and 400 were sufficient to provide robust fragmentation (~40–60% conversion of precursor). In all cases, the fragmentation was qualitatively similar to HCD with some unique fragmentation through radical channels observed in UVPD. In Figure 7, we show the comparison of UVPD and HCD fragmentation for DHP-theophylline, which was used as a model compound to explore the assumptions based on results from caffeine and theobromine. Importantly, several unique UVPD fragments were the result of fragmentation across the ring not observed by HCD, including the retention of the dihydroxypropyl group. Given the shift in the UV absorbance of hypoxanthine reducing the absorbance around 200–220nm, we expected the fragmentation induced by UVPD to be poor for this compound, but significant for the other three xanthine-related compounds studied.

Figure 7. Comparison of the UVPD and HCD fragmentation of DHP-Theophylline.



When considering the pulse count required for each xanthine compound studied, all compounds typically required only 200-400 pulses (total reaction time 100-200 msec) to obtain fragmentation except for hypoxanthine which showed almost no fragmentation by UVPD after more than 800 pulses (400msec). This falls in line with our observation of the shift in absorbance for hypoxanthine away from a maximal absorbance near the wavelength of the laser (213nm). This would further support that optimizing the wavelength and energy per pulse could provide improved results for flavonoids as well.

CONCLUSIONS

- UVPD can generate fragmentation which is similar to HCD.
- The kinetics of fragmentation are not equivalent between UVPD and HCD (the resulting fragmentation ratios do no match)
- UVPD provides unique fragmentation for he compounds studied which may be useful for structure determination / substructure identification.
- "Tuneable" energy per pulse may be required to assure fragmentation primarily driven by photon absorption versus more distributed vibrational fragmentation
- Further studies with differing wavelengths could provide even more useful and diagnostic fragmentation.

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