Application note | 000531

Mass spectrometry

Impurity profiling of mycophenolate mofetil using an Orbitrap Exploris 120 mass spectrometer and Vanquish Horizon UHPLC combined with Compound Discoverer software

Authors

Kate Comstock, Min Du

Thermo Fisher Scientific, San Jose, CA

Keywords

Benchtop Orbitrap mass spectrometer, Orbitrap Exploris 120 MS, high resolution accurate mass, HRAM, Vanquish UHPLC, pharma impurity profiling, data processing software, small molecule structure identification

Goal

Describe a workflow, comprised of an ultra-high performance liquid chromatography system (UHPLC) coupled to an HRAM-MS system and employing advanced data mining software, that provides confident impurity identification and structure elucidation of mycophenolate mofetil.

Application benefits

- Powerful benchtop Orbitrap™ mass spectrometer for drug impurity analysis
- HRAM full MS and MS^2 data with rapid polarity switching
- Easy-to-use interface for instrumental control and method setup
- Advanced data processing software

Introduction

Drug substance and drug product impurity profiling is an important part of drug R&D and is required by health agencies globally. Knowledge of a drug candidate's impurity profile and chemical structures is essential for drug toxicity assessment to ensure drug efficacy and safety. UHPLC-HRAM MS has been routinely used for drug impurities and degradation product identification and structure characterization in pharmaceutical research and development.

Mycophenolate mofetil (MMF), brand name CellCept®, is the prodrug of immunosuppressant mycophenolate acid (MPA). MMF is used extensively in transplant medicine for treatment of organ transplant rejection and autoimmune diseases.

In this study, a comprehensive workflow for rapid and confident impurity detection and structure elucidation of mycophenolate mofetil has been developed by employing a Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer coupled with a Thermo Scientific™ Vanquish™ Horizon UHPLC system (Figure 1). Thermo Scientific™ Compound Discoverer™, a small molecule identification software, was used for data processing.

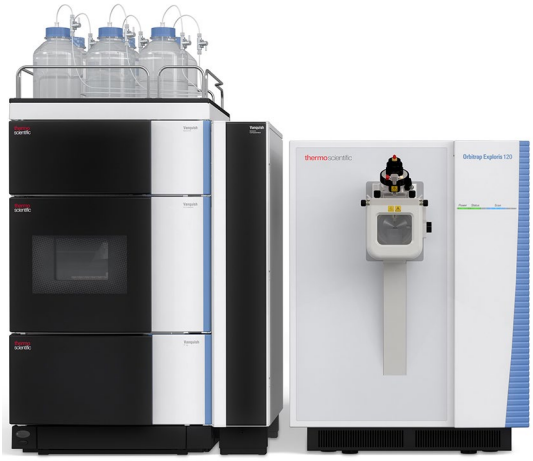
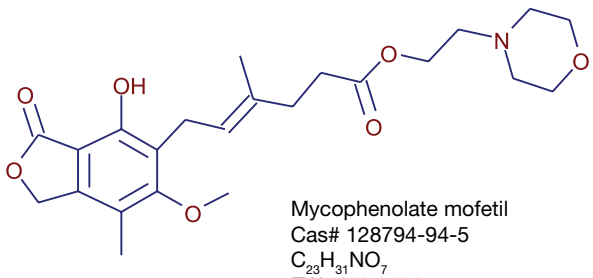


Figure 1. Orbitrap Exploris 120 mass spectrometer coupled with a Vanquish Horizon UHPLC system

Experimental

Reagents and consumables

- Thermo Scientific™ Water, UHPLC-MS grade (P/N W8-1)
- Thermo Scientific™ Acetonitrile, UHPLC-MS grade (P/N A9561)
- Fisher Chemical™ Formic acid, Optima™ LC-MS grade (P/N A117-10X1AMP)
- Sigma-Aldrich, Ammonium formate, (P/N 516961-100G)
- Mycophenolate mofetil from Sigma-Aldrich (P/N SML0284-10MG)



Mycophenolate mofetil
Cas# 128794-94-5
$C_{23}H_{31}NO_7$
FW. 433.21005
$(M+H)^+$ 434.21733

Figure 2. Mycophenolate mofetil, CAS# 128794-94-5

Sample preparation

Mycophenolate mofetil stock solution at 1.0 mg/mL in acetonitrile was prepared by dissolving 1.0 mg in 1 mL acetonitrile. The working solution for LC-MS analysis was 0.25 mg/mL in water with 25% ACN.

Liquid chromatography

Chromatographic separations were carried out on the Thermo Scientific™ Vanquish™ Horizon UHPLC system consisting of the following modules:

- Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A)
- Thermo Scientific™ Vanquish™ Split Sampler HT (P/N VH-A10-A)
- Thermo Scientific™ Vanquish™ Column Compartment (P/N VH-C10-A)
- Thermo Scientific™ Vanquish™ Diode Array Detector FG (P/N VF-D11-A)

A Thermo Scientific™ Hypersil GOLD™ VANQUISH™ C18 UHPLC column (2.1 × 100 mm, 1.9 µm, P/N 25002-102130-V) was used with the gradients specified below at a flow rate of 0.4 mL/min and column temperature of 50 °C. The injection volume was 5 µL. Mobile phase was composed of: (A) H_2O/0.1% formic acid/10 mM ammonium formate and (B) acetonitrile/0.1% formic acid.

LC gradient

Time (min)	B%
0	10.0
1.0	10.0
3.0	20.0
12.0	40.0
15.9	95.0
18.5	95.0
18.6	10.0
20.0	10.0

Mass spectrometry

The mass spectrometry analyses were carried out on an Orbitrap Exploris 120 mass spectrometer (P/N BRE725531) equipped with a Thermo Scientific™ OptaMax™ NG ion source.

The data was acquired using full scan MS followed by Top-4 $ddMS^2$ with polarity switching. An EASY-IC internal mass calibration was employed to ensure high mass accuracy throughout. The acquisition methods were set up using the method template in the method editor (Figure 3).

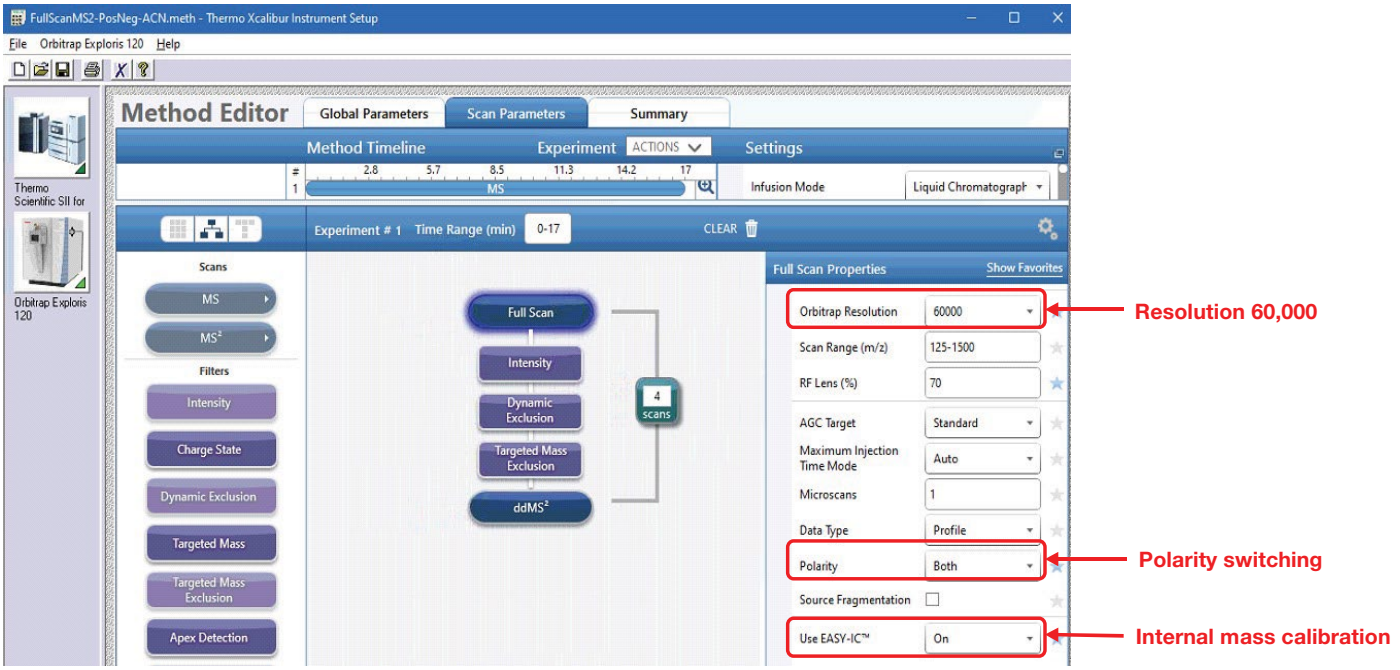


Figure 3. Easy MS method setup in method editor: Full scan followed by four DDA with polarity switching and internal calibration

Source parameters

Parameter	Settings
Ion source	OptaMax NG electrospray ion source
Ionization mode	ESI positive/negative
Scan range (Full MS) (*m/z*)	125–1,500
Spray voltage (kV)	+3.5 (positive)/-2.5 (negative)
Ion transfer tube temp (°C)	320
S-lens RF level	70.0
Vaporizer temp (°C)	400
Sheath gas (Arb)	40
Aux gas (Arb)	10
Sweep gas (Arb)	2

MS method parameters

Parameter	Settings
Resolution (Full Scan/MS^2)	60,000/15,000
AGC target	Standard
Max injection time (Full Scan/MS^2)	Auto
Data-dependent MS^2 (ddMS^2) acquisition	Top 4 for data acquisition using positive/negative polarity switching; Top 4 for data acquisition using positive mode only
Isolation window (*m/z*)	1.6
Normalized HCD (%)	20, 30, 45

Results and discussion

High-quality full scan/DDA MS^2 data with polarity switching

To capture impurities with different ionization preferences, data acquisition using rapid polarity switching is necessary for drug impurity profiling. In this study, data was acquired using full MS followed by top 4 DDA MS^2 at resolution 60,000 (full MS) and 15,000 (MS^2), respectively, with polarity switching. The high scan speed of the Orbitrap Exploris 120 mass spectrometer enabled rapid data acquisition with a duty cycle of approximately 1 second for a total of 10 scan events (Figure 4). As a result, information-rich HRAM full scan and MS^2 fragments of both polarities were obtained in a single run.

Figure 5 shows the zoomed-in view of an MS total ion chromatogram with polarity switching and UV spectrum of MMF.

The results show that in the presence of high concentration parent compound, the low abundant impurities were detected with confidence owing to the Orbitrap Exploris 120 mass spectrometer's high dynamic range, high sensitivity, and high mass accuracy. These capabilities are essential for API impurity identification and drug product degradation profiling.

HRAM positive/negative switching acquisition provided confirmative information for formula mass and elemental composition of impurity at RT 5.97 min. In addition, the complementary polarity unique fragments aided in the definitive structure identification of this impurity (Figure 6).

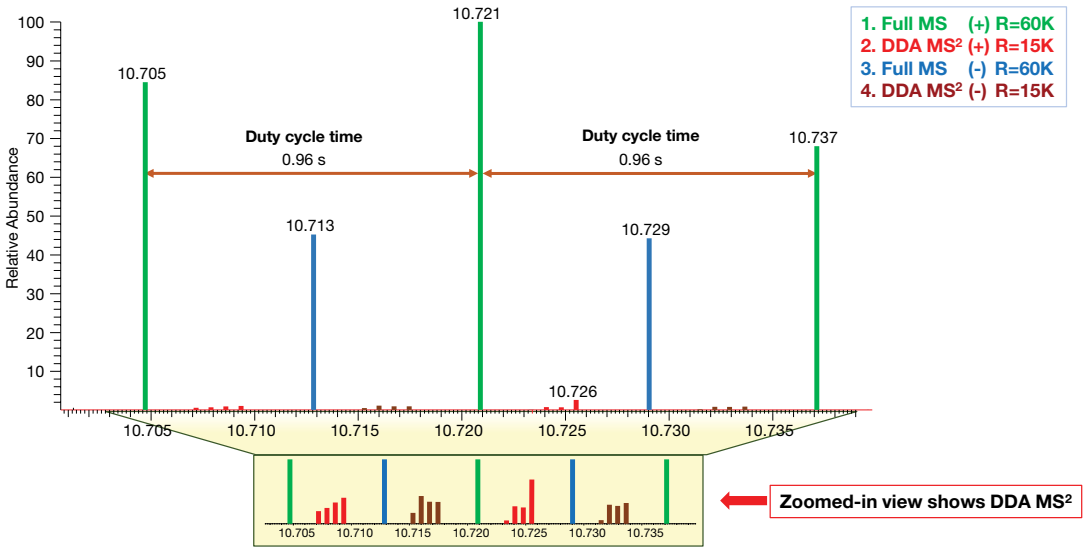


Figure 4. Fast scan speed for high resolution full Scan-DDA MS² with polarity switching

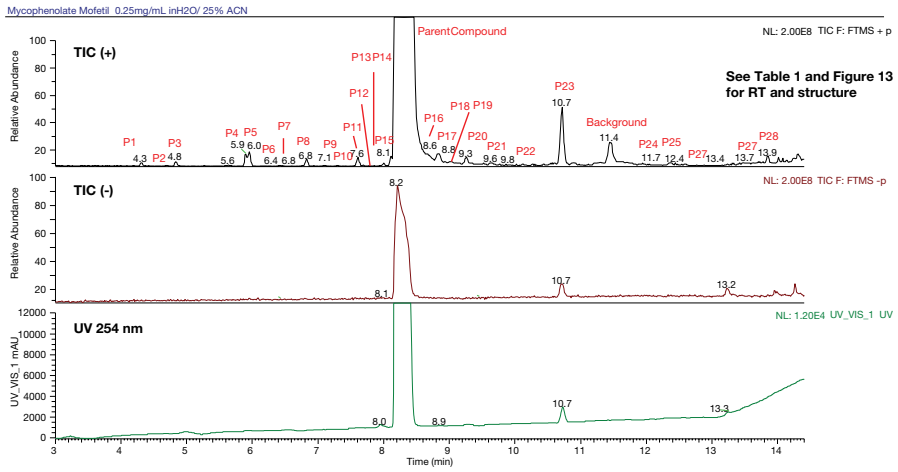


Figure 5. LC-MS chromatogram of mycophenolate mofetil

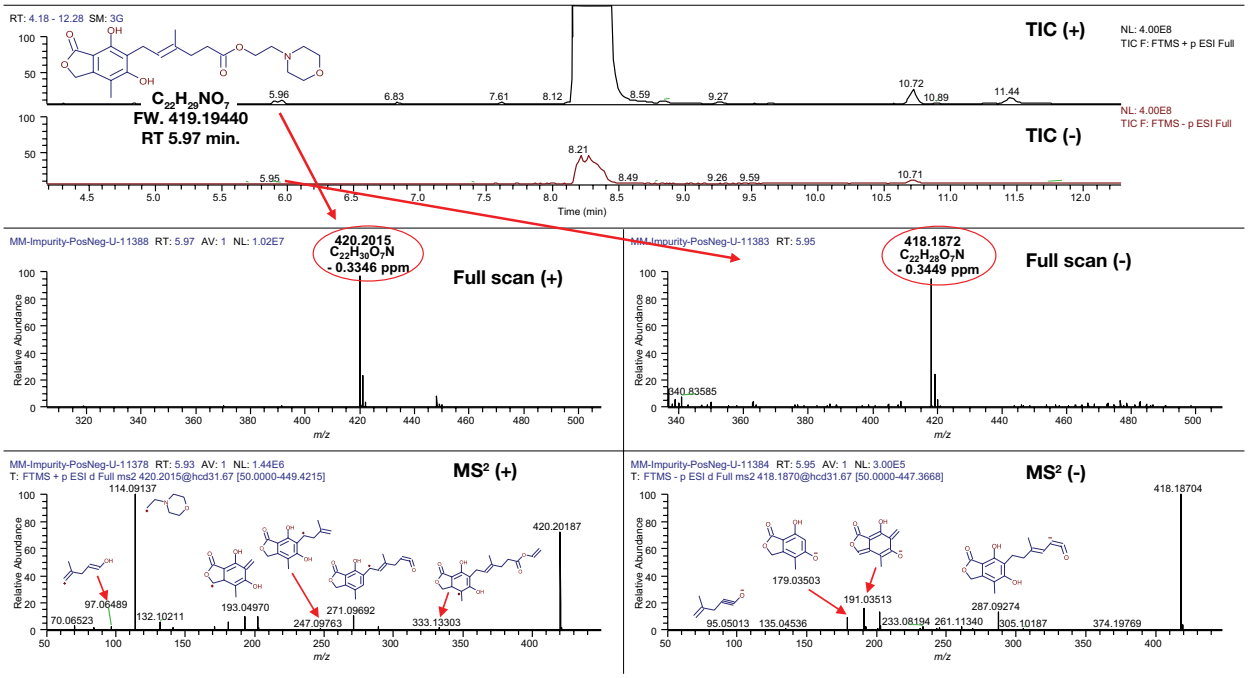


Figure 6. HRAM full scan- HCD MS² with polarity switching in a single run

HRAM full scan and MS^2 data are needed for structure elucidation. In this study, results processed using Compound Discovered software show that there are four impurities that have the same $(M+H)^+$ 450.2121 and the same elemental composition of $C_{23}H_{31}NO_8$, but eluted at different times. Based on their MS^2 fragments, utilizing the software's unique features, the structures of these impurities were proposed with confidence (Figures 7a and 7b).

Data processing using Compound Discoverer 3.2 software

With HRAM full scan and HCD DDA MS^2 data acquisition, data processing software with an effective data mining tool plays an important role for impurity identification and structure characterization.

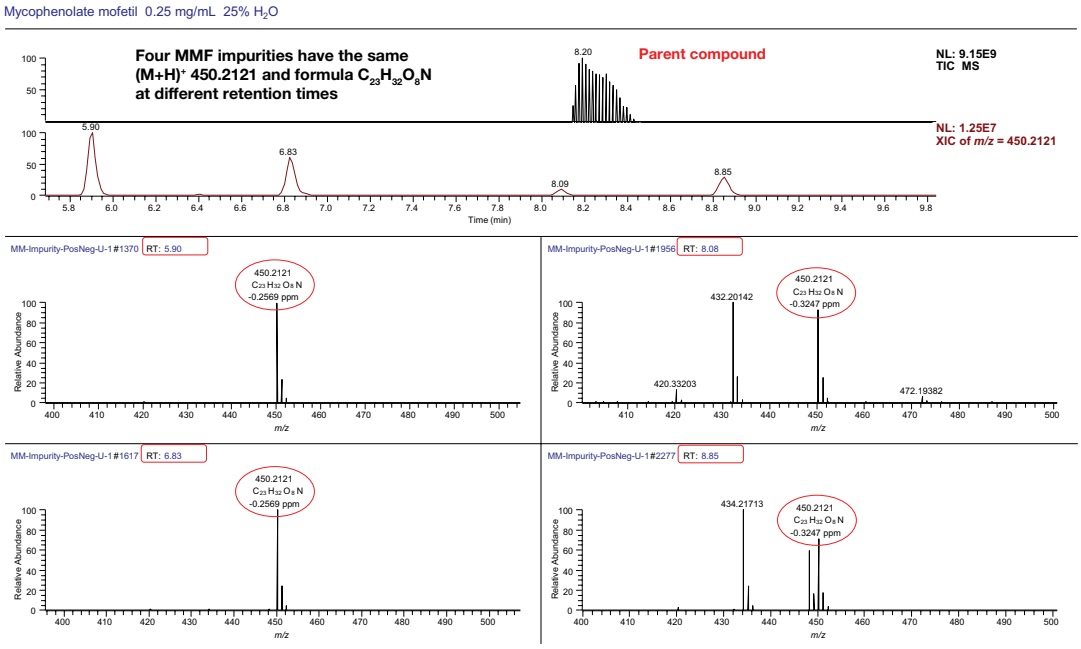


Figure 7a. HRAM full scan and HCD MS^2 data for impurity structure determination

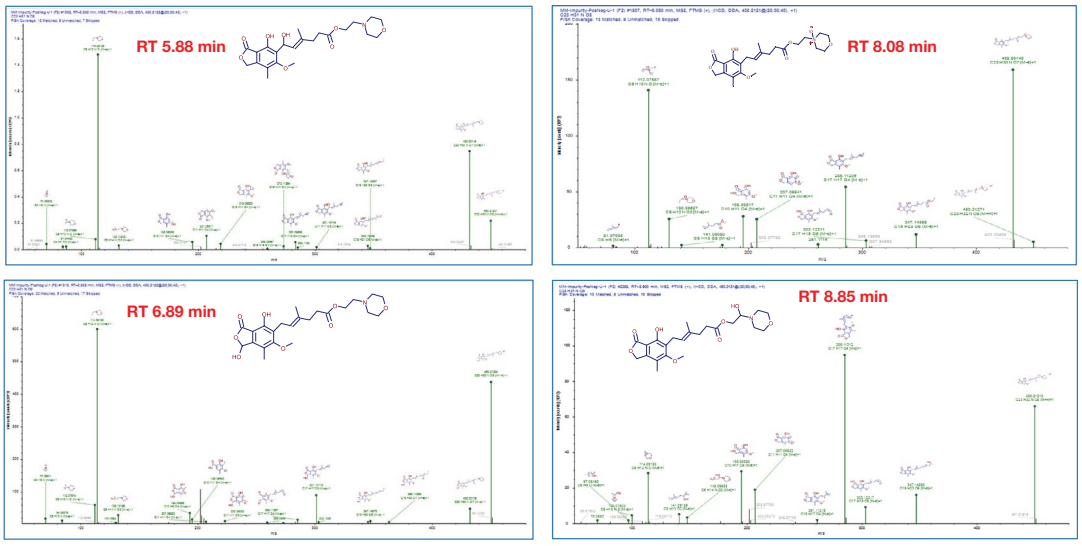


Figure 7b. HCD MS^2 fragments facilitate impurity structure elucidation

In this study, the HRAM full scan and HCD DDA MS^2 data was processed using Compound Discoverer 3.2 software, a small molecule structure analysis software that employs a flexible and customizable node-based processing workflow.

Compound Discoverer 3.2 software processes high-resolution accurate-mass (HRAM) data exclusively, because the advanced algorithm requires accurate mass and isotope pattern for component extraction and elemental composition prediction. Based on the predicted formula, accurate mass, and the MS^n fragment spectra, the node-based workflow conducts targeted and untargeted compound identification through database search and user-defined approaches utilizing various application-specific nodes. The known structure verification and unknown structure proposal are carried out using the Structure Proposal and FISh Scoring (FISh = Fragment Ion Search) features. The validity of known compound and proposed unknown structures is then evaluated by its FISh Coverage score based on the number of matched and unmatched fragment ions.

In this study, the processing workflow was built by following the step-by-step New Study and Analysis Wizard using the pre-defined workflow template Impurity ID Related and Unknown (Figure 8).

This processing workflow captures expected and unexpected impurities using targeted and user-defined approaches, as well as unknown impurities based on relative abundance vs. blank.

Targeted approach - Identifying impurities through the Generate Expected Compounds node

I. The Generate Expected Compounds node detects the impurities generated by common reactions from parent compound. These common reactions are listed in Compound Discoverer 3.2 software and include dealkylation, diarylation, oxidation, reduction, hydration, dehydration, desaturation, acetylation, methylation, etc. In addition, users can add any modifications that are specific to the individual parent compound structure.

User-defined approach - Identifying impurities through the Compound Class Scoring and Pattern Scoring nodes

I. The Compound Class Scoring node detects any impurities that are generated not from the listed common reactions, but those that share the same MS^2 fragment(s) with the parent compound. The Compound Class list containing parent compound MS^2 fragments is required for the Compound Class Scoring node.

II. The Pattern Scoring node finds compounds that have user-defined unique isotopic patterns. This feature is especially useful for radiolabeled study data processing and compounds containing sulfur, chlorine, bromine, etc.

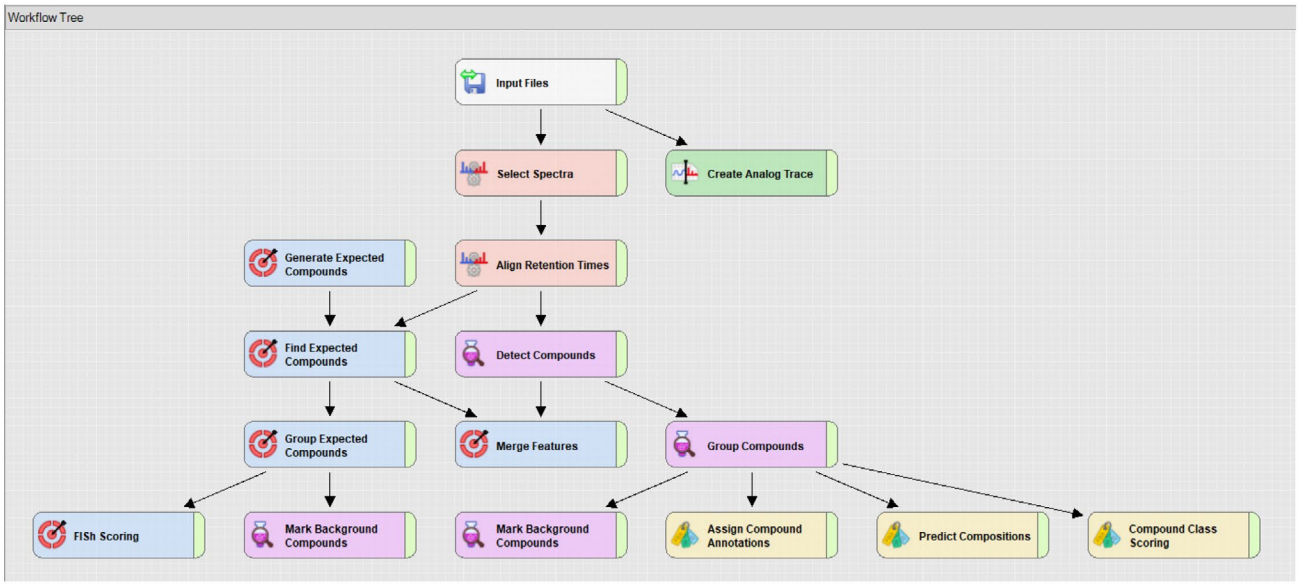


Figure 8. Compound Discoverer Impurity ID processing workflow tree

Unknown impurity identification – *de novo* structure elucidation

I. Unknown impurities are the impurities for which structures do not fall in the expected nor user-defined categories. It includes impurities from starting material, reagents, catalysts, etc. These impurities are detected based on their relative abundance between sample vs. blank. Their structure elucidations rely upon the predicted formula, accurate mass, and fragmentation data for structure elucidation.

The processed results are shown in the Processing Result view (Figure 9).

The Compounds table contains comprehensive information on all identified compounds. The XIC chromatographic and full scan, MS^2 spectra properties of each impurity can be inspected through the interactive Chromatograms and Mass Spectrum views. The Related Tables contain the related information for each identified compound.

The Expected Compounds table listed only the impurities generated from parent compound via common reaction. It includes Formula, Transformation, Composition Change, FISh Coverage score (Figure 10). For each identified expected impurity, the full scan mass spectrum shows the accurate mass of assigned elemental formula with color-coded fine isotopic pattern confirmation. The automatically annotated and color-coded MS^2 fragments indicate the fragments that either match or have mass shift compared with corresponding parent MS^2 fragment(s).

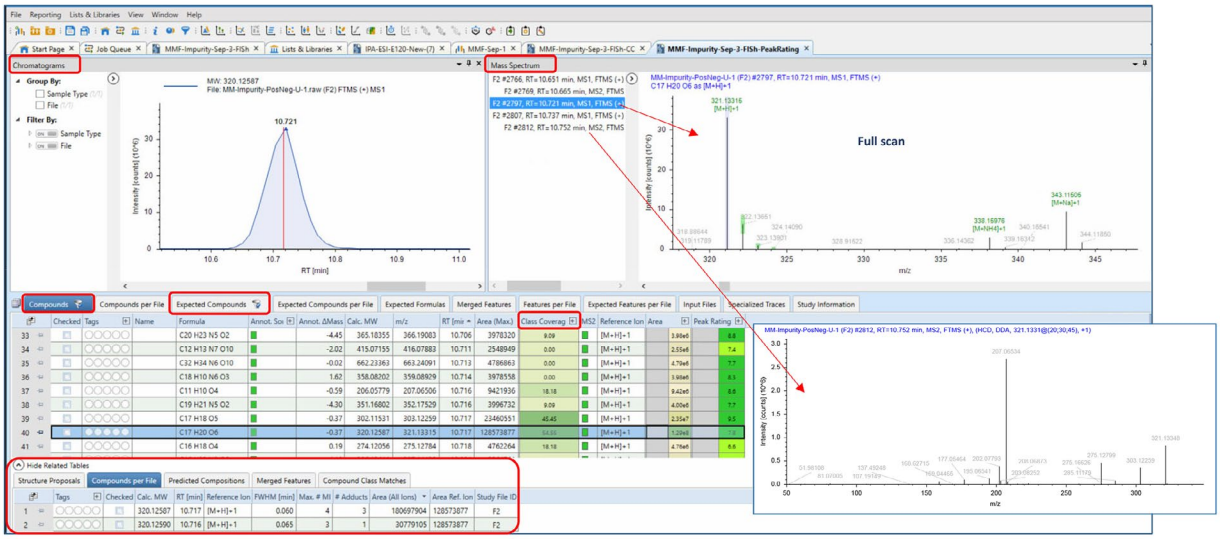


Figure 9. Compound Discoverer 3.2 software Processing Result view

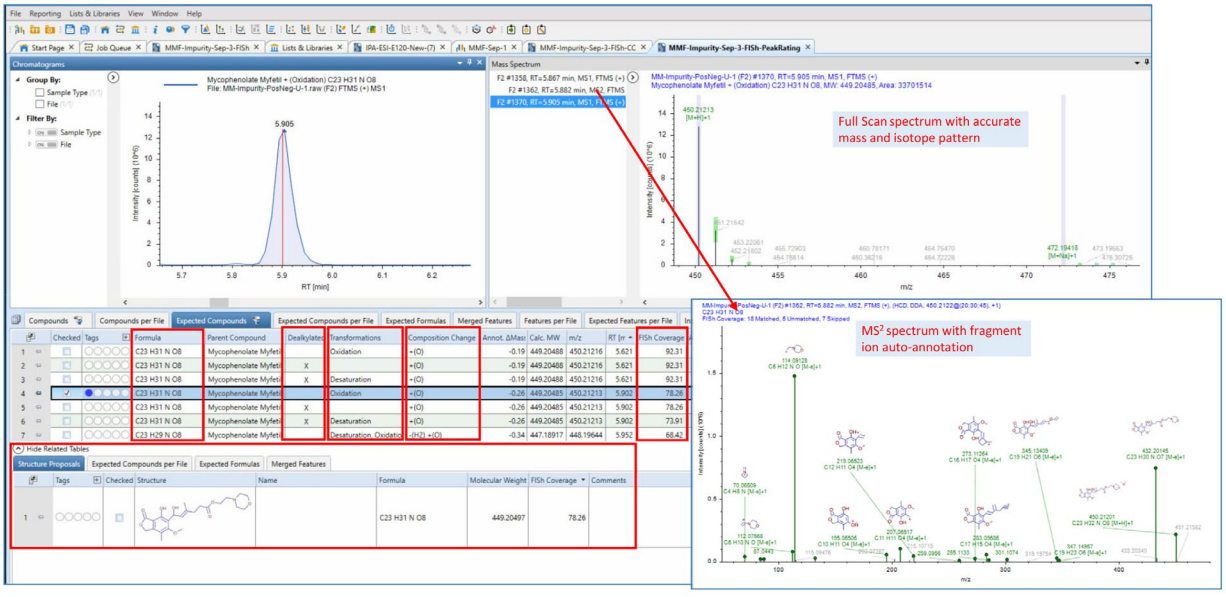


Figure 10. Expected Compounds table

The Transformations table displays the proposed possible modification (if available) (Figure 11a).

Based on the accurate mass, formula, and fragment annotations in the Expected Compounds table, the impurity structures were proposed in the Structure Proposal tab, followed by FISh Scoring, which conducts in silico fragment prediction for the proposed structure, annotates matching fragment ions, and generates FISh Coverage scores that indicate the plausibility of the detected compounds.

To inspect the fragment difference between parent and impurity, the parent fragment spectrum was selected as reference in the Mass Spectrum view to display the intuitive mirror plot of MS^2 spectra of parent and selected impurity. The mirror plot with fragment annotation revealed the site of modification: the green lines represent the unchanged fragments that match with the parent compound fragments, and the blue lines represent the fragments with modification. The impurity structure was proposed based on the fragment difference (Figure 11b).

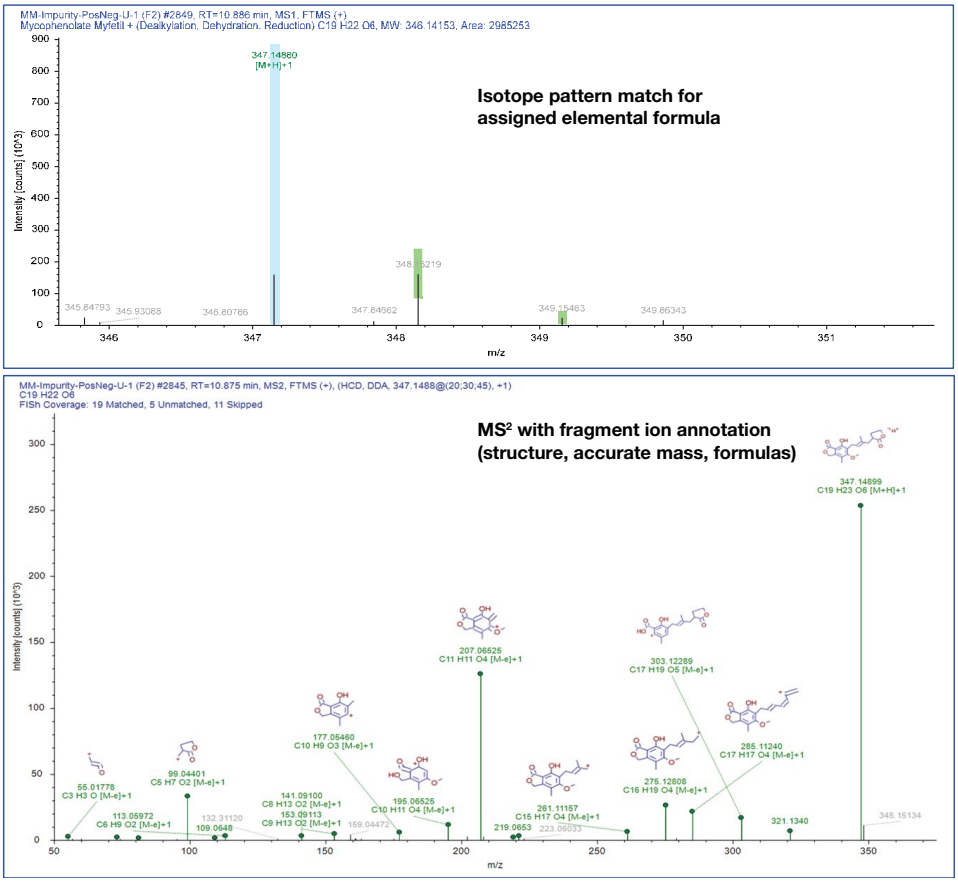


Figure 11a. Full scan and MS^2 spectra of identified expected compound

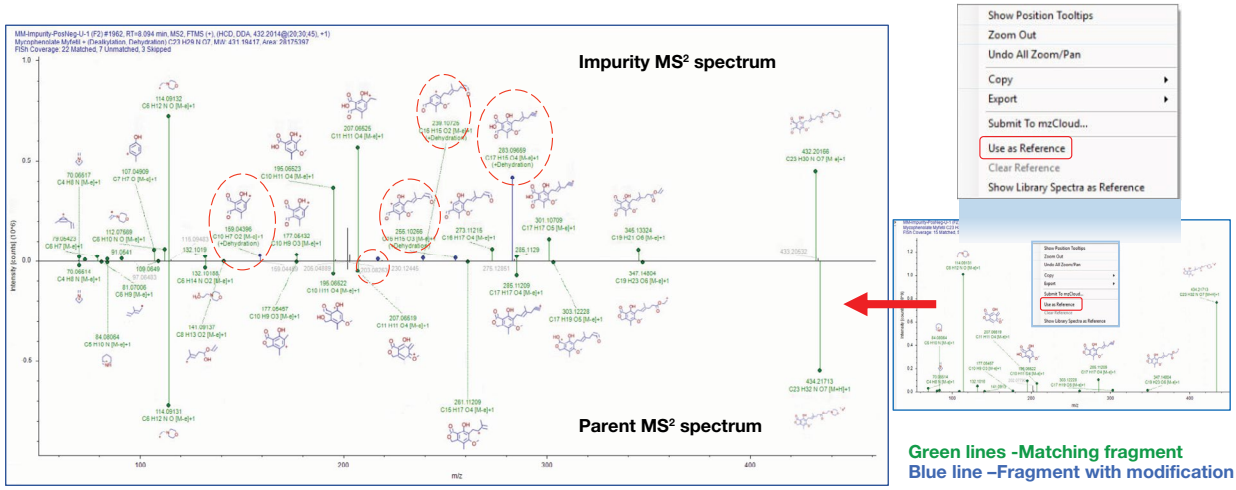


Figure 11b. Mirror plot and auto annotation of MS^2

The impurities do not fall into the expected compound category but share the same MS^2 fragment(s) with the parent compound identified by the Compound Class feature. The Compound Coverage column in the main Compounds table displays the percentage of matching fragments. The matching fragments can be viewed by clicking on the Compound Class Matches tab in Related Tables or from the MS^2 spectra mirror plot with parent. Based on the accurate mass, predicted formula, and MS^2 spectrum, the impurity's structure was proposed using the Structure Proposal feature, and FISh Scoring was carried out to check the validity of proposed structure (Figure 12).

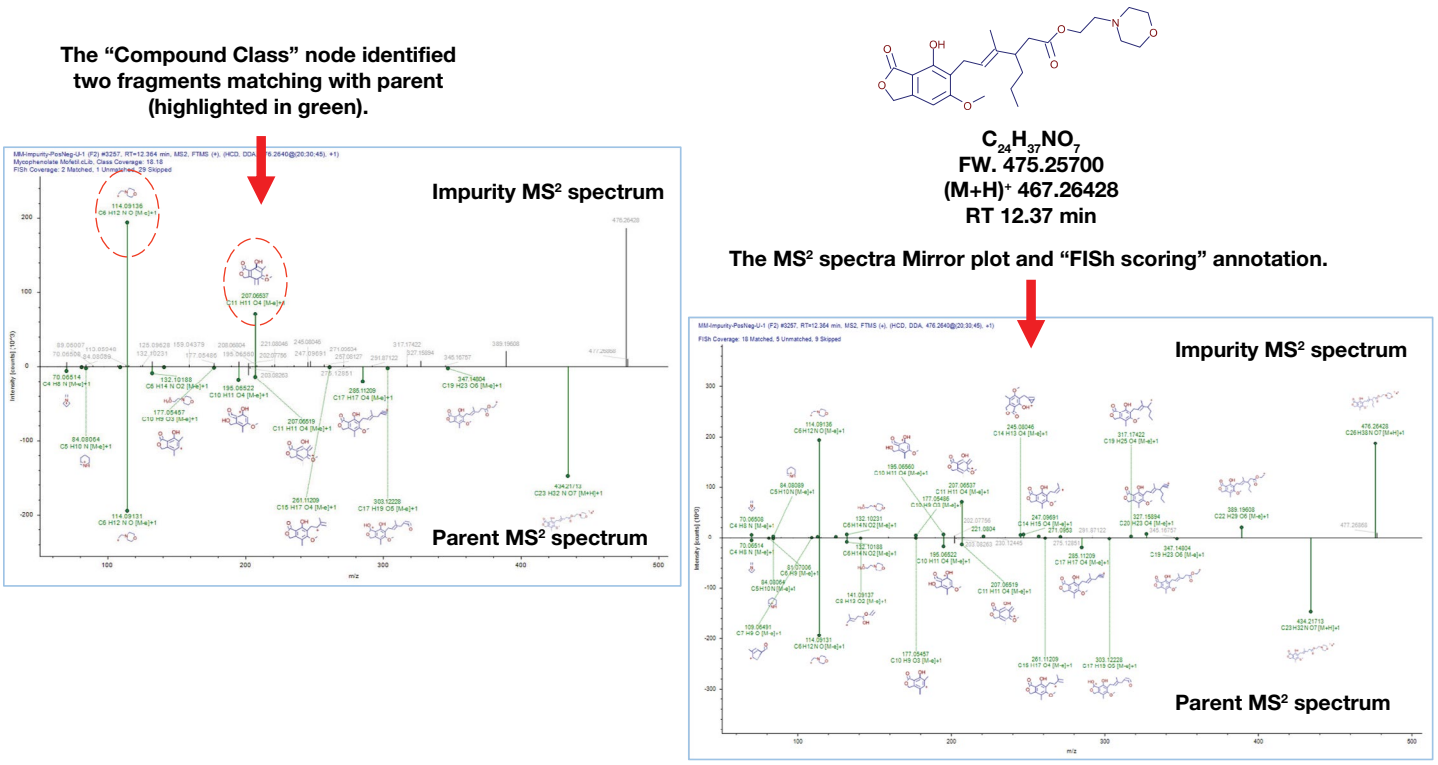


Figure 12. Impurity identification through Compound Class feature

Result report from Compound Discoverer 3.2 software data processing

The processed impurity results were exported and summarized in Table 1. Tag D indicates the impurities identified through Expected Compounds, and Tag A indicates the impurities identified through Compound Class. The proposed structures are shown in Figure 13.

The Compound Discoverer 3.2 data processing report can be generated using the template Expected Compounds with Structure with Graphs, which includes structure, full MS with isotope pattern, and annotated MS^2 spectrum.

In addition to the ready-to-use report templates, Compound Discoverer 3.2 software also provides a tool kit to generate custom report formats.

Table 1. Impurities identified using Compound Discoverer software for data processing

Peak #	Tags	RT [min]	Formula	Calculalted MW	*m/z*	Delta mass [ppm]	By compound class	By expected compounds
1	A	4.31	$C_{29}H_{42}N_2O_9$	562.28880	563.29608	-0.40	A	
2	A	4.69	$C_{29}H_{42}N_2O_8$	546.29393	547.30121	-0.34	A	
3	A	4.84	$C_{29}H_{42}N_2O_9$	562.28874	563.29602	-0.51	A	
4	D	5.90	$C_{23}H_{31}NO_8$	449.20485	450.21213	-0.26		D
5	D	5.96	$C_{22}H_{29}NO_7$	419.19426	420.20154	-0.34		D
6	D	6.43	$C_{23}H_{33}NO_8$	451.22051	452.22778	-0.24		D
7	A	6.73	$C_{23}H_{29}NO_8$	447.18923	448.19650	-0.20	A	
8	D	6.83	$C_{23}H_{31}NO_8$	449.20485	450.21213	-0.26		D
9	D	7.11	$C_{23}H_{29}NO_7$	431.19420	432.20148	-0.47		D
10	D	7.30	$C_{23}H_{29}NO_8$	447.18920	448.19647	-0.27		D
11	D	7.61	$C_{22}H_{29}NO_7$	419.19426	420.20154	-0.34		D
12	D	7.69	$C_{17}H_{18}O_6$	318.11022	319.11749	-0.38		D
13	D	8.00	$C_{17}H_{18}O_6$	318.11024	336.14407	-0.32		D
14	D	8.09	$C_{23}H_{31}NO_8$	449.20485	450.21213	-0.26		D
15	D	8.12	$C_{23}H_{29}NO_7$	431.19417	432.20145	-0.54		D
	MMF	8.20	$C_{23}H_{31}NO_7$	433.21733	434.21710	-0.53	Parent Compound	
16	D	8.61	$C_{24}H_{34}N_2O_6$	446.24147	447.24875	-0.48		D
17	D	8.83	$C_{24}H_{33}NO_7$	447.22554	448.23282	-0.36		D
18	D	8.85	$C_{23}H_{31}NO_8$	449.20482	450.21210	-0.33		D
19	A	9.04	$C_{27}H_{38}N_2O_8$	518.26262	519.26990	-0.38	A	
20	D	9.26	$C_{25}H_{35}NO_8$	477.23598	478.24326	-0.60		D
21	A	9.52	$C_{26}H_{37}NO_8$	491.25176	492.25903	-0.33	A	
22	D	10.72	$C_{17}H_{20}O_6$	320.12587	321.13315	-0.37		D
23	D	10.88	$C_{19}H_{22}O_6$	346.14153	347.14880	-0.32		D
24	D	11.72	$C_{23}H_{33}NO_6$	419.23061	420.23788	-0.43		D
25	A	12.37	$C_{26}H_{37}NO_7$	475.25673	476.26401	-0.57	A	
26	D	12.59	$C_{23}H_{29}NO_8$	447.18923	448.19650	-0.20		D
27	A	13.72	$C_{28}H_{39}NO_7$	501.27254	502.27982	-0.23	A	
28	D	13.85	$C_{18}H_{22}O_6$	334.14149	335.14877	-0.44		D

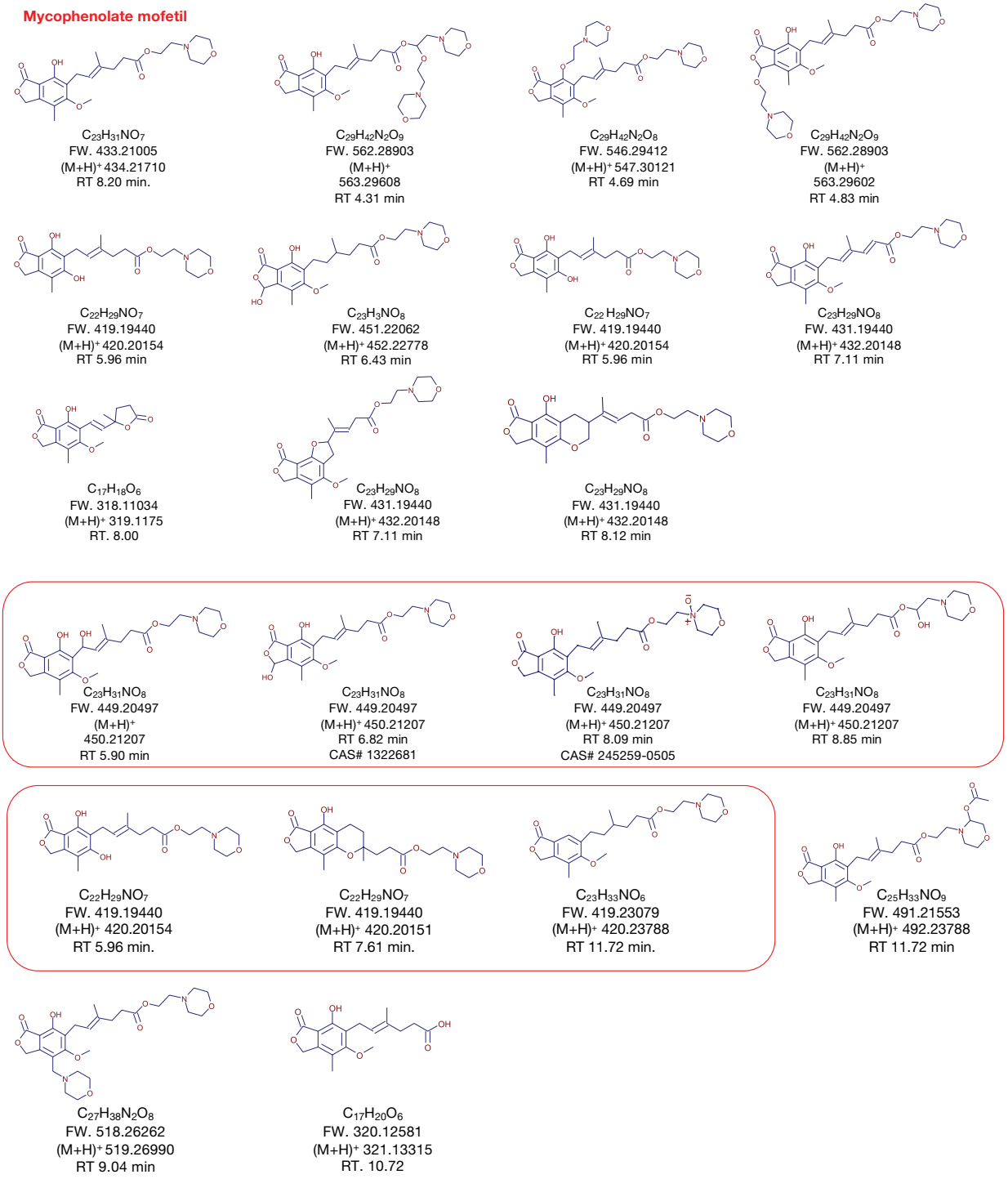


Figure 13. Proposed structures of mycophenolate mofetil API impurities

Conclusion

In this application note, we have described a workflow for impurity profiling of mycophenolate mofetil using a benchtop Orbitrap Exploris 120 mass spectrometer and a Vanquish Horizon UHPLC system combined with Compound Discoverer 3.2 software.

The following key features of the hardware and software ensured high-quality HRAM data and subsequent data processing for confident impurity identification and structure characterization:

- The Orbitrap Exploris 120 mass spectrometer's high sensitivity, high dynamic range, and high mass accuracy enabled confident impurity profiling, especially for accurate identification of low abundant, trace-level impurities in the presence of an excessive amount of parent compound.
- HRAM full scan and HCD MS^2 spectra with polarity switching data acquisition generated a high-quality dataset in a single run.
- Utilizing the full scan and MS^2 spectra, Compound Discoverer 3.2 software's advanced algorithm and versatile features enabled confident impurity ID and structure elucidation.

This workflow, utilizing a high-performance, easy-to-use UHPLC-HRAM MS system and advanced data processing software, improves overall quality and efficiency for routine impurity identification and structure characterization. This workflow is well suited for other small molecule structure analysis applications, such as metabolite ID and E&L analysis.

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