An Effective Workflow for Impurity Analysis Incorporating High Quality HRAM LCMS & MSMS with Intelligent Automated Data Mining

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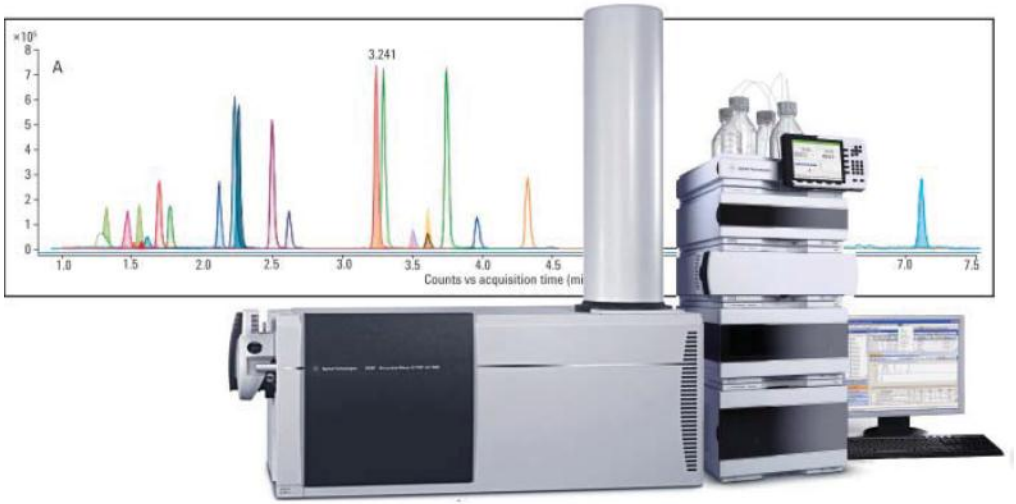
Jim Lau, Ph.D.



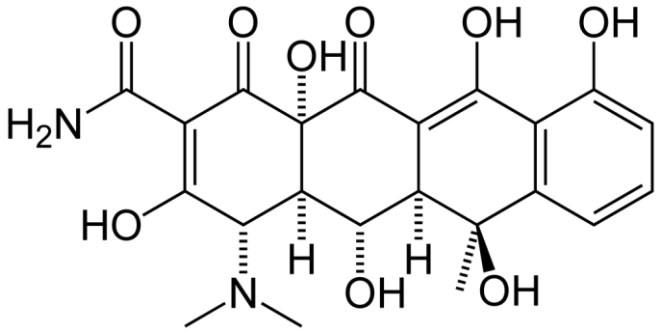
Typical Method Conditions: 1260 UHPLC

UHPLC: Agilent 1260 Binary pump, well plate autosampler, thermostatted column compartment

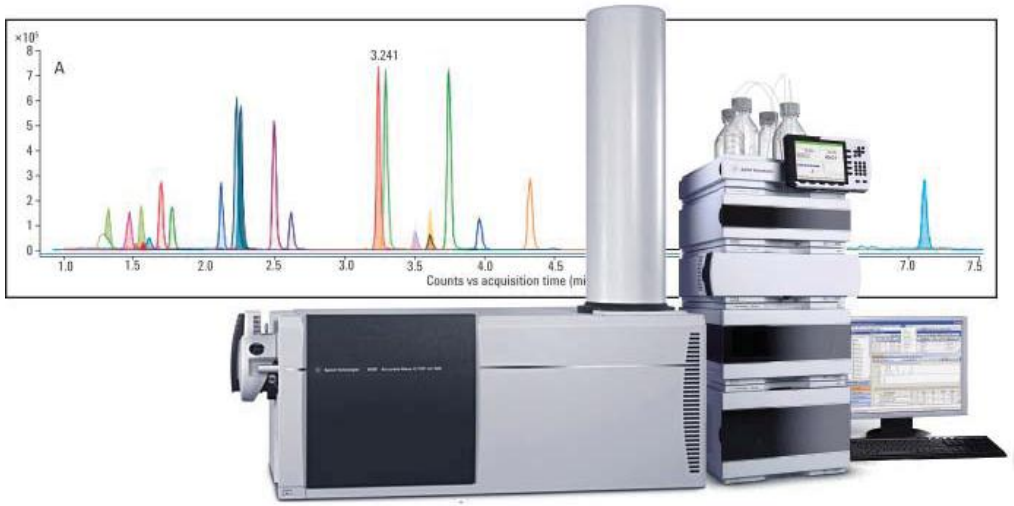
Column:	Agilent Zorbax Eclipse Plus C-18, 1.8 um, 2.1 mm x 50 mm
Column temperature:	40°C
Injection volume:	1 or 2 ul Oxytetracycline standard
Autosampler temp:	4 ºC
Needle wash:	flushport (water:methanol 50:50), 3 seconds
Mobile phase:	A = 0.05% Trifluoroacetic acid in H2O
	B = 0.045% Trifluoroacetic acid in ACN
Flow rate:	0.5ml/min
Gradient:	Time (min) %B
	0.00 10
	7.50 50

Impurity Analysis of Oxytetracycline and H20 Stressed (30 min at 90 deg C)

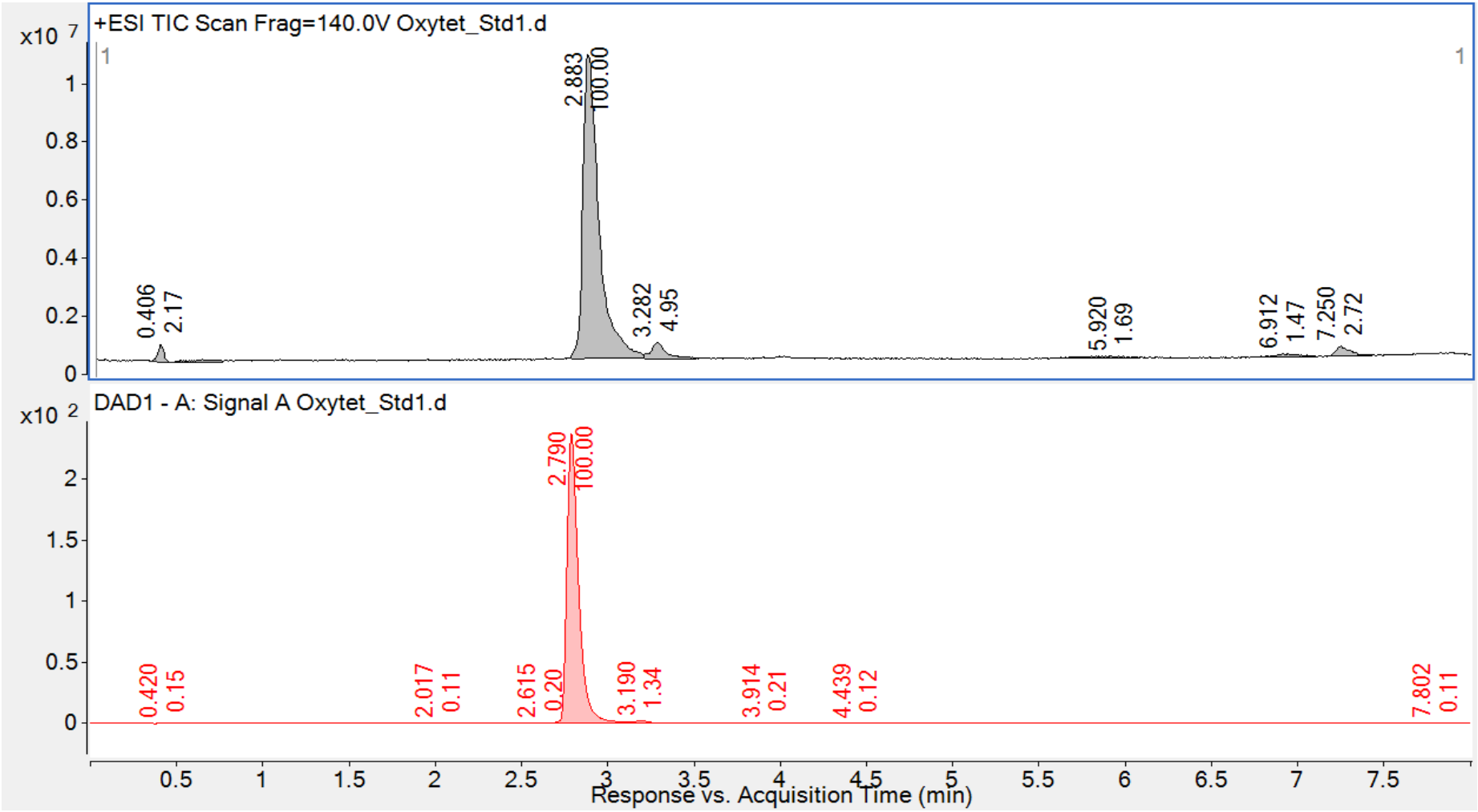
Oxytetracycline



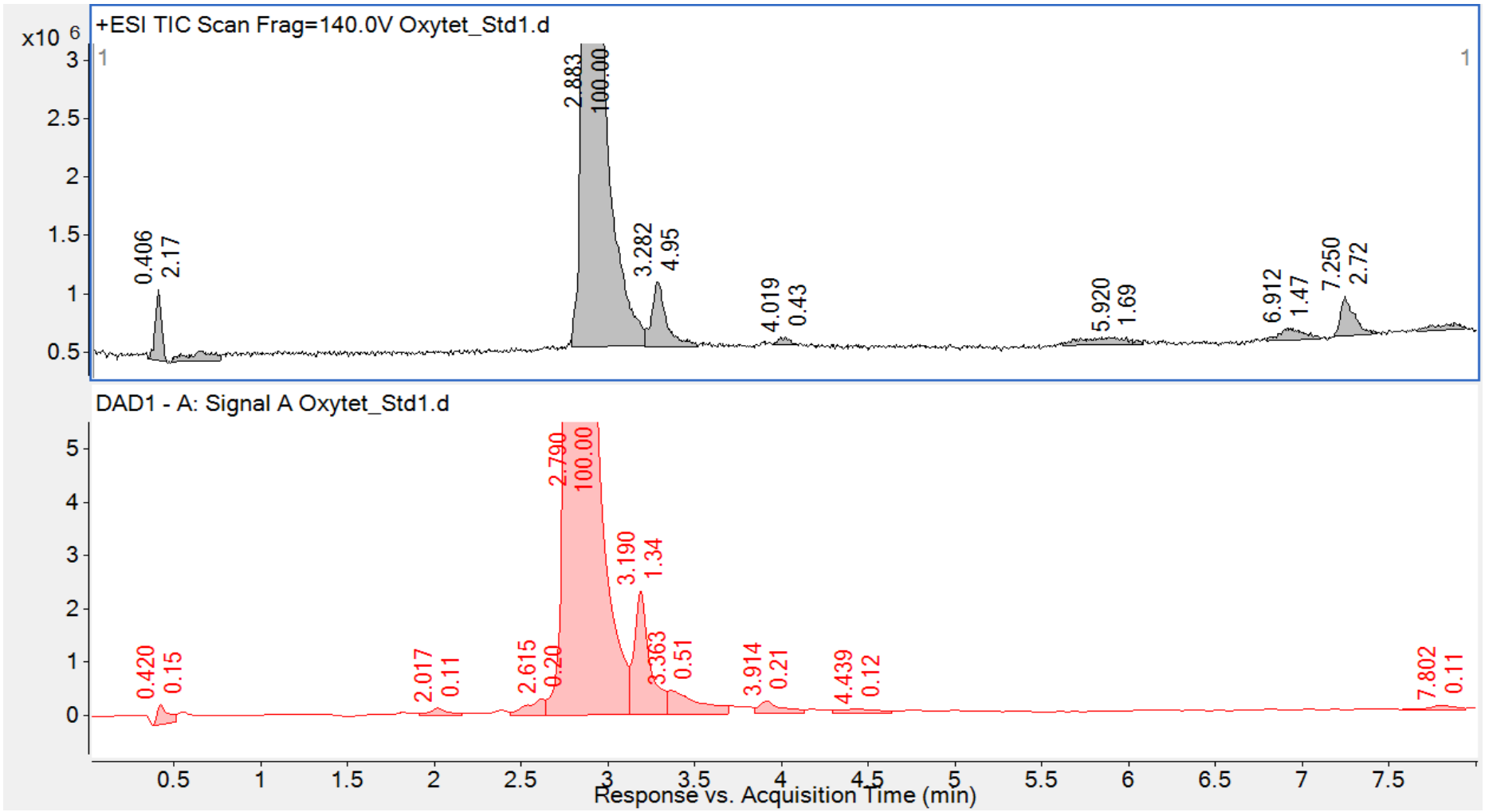
trade name: Terramycin
$C_{22}H_{24}N_2O_9$
460.148183



Pos ESI LC/MS TIC and UV Chromatograms of Standard Oxytetracycline:



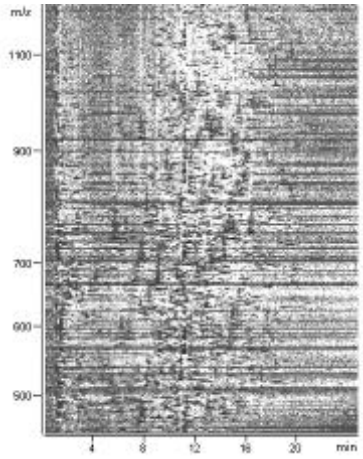
Expanded TIC and UV Chromatograms of Standard Oxytetracycline:

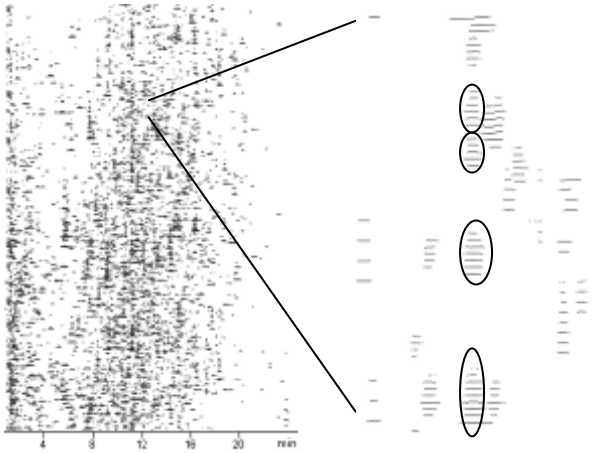


1st Step – Use MassHunter Qual software to find all components in the sample using the automated MFE (Molecular Feature Extraction) chromatographic deconvolution algorithm.

Molecular Feature Extractor (MFE)

Transforming Accurate MS Data to Chemical Information





species	RT	m/z	mass	abund.
M+3H	13.248	921.5025	2761.4856	356764
M+3H+1	13.247	921.8373	2761.4874	548320
M+3H+2	13.248	922.1722	2761.4897	476780
M+3H+3	13.249	922.5072	2761.4924	256680
M+3H+4	13.249	922.8416	2761.4934	117149
M+3H+5	13.251	923.1744	2761.4892	45705
M+3H+6	13.252	923.5058	2761.4810	15721
M+3H+7	13.246	923.8387	2761.4775	4492
M+3H+8	13.263	924.1649	2761.4535	677
M+2H+Na	13.248	928.8293	2761.4842	29521
M+2H+Na+1	13.249	929.1643	2761.4863	46140
M+2H+Na+2	13.247	929.4957	2761.4781	43276
M+2H+Na+3	13.247	929.8283	2761.4737	33217
M+2H+Na+4	13.258	930.1614	2761.4706	23203
M+2H+Na+5	13.285	930.5000	2761.4842	11741
M+2H+Na+6	13.277	930.8343	2761.4846	3970
M+2H+K	13.247	934.1483	2761.4673	8064
M+2H+K+1	13.241	934.4889	2761.4863	13793
M+2H+K+2	13.244	934.8196	2761.4759	11070
M+2H+K+3	13.250	935.1566	2761.4847	5695
M+2H+K+4	13.247	935.4933	2761.4926	3721
M+2H+K+5	13.280	935.8045	2761.4237	1182
M+H+2Na	13.238	936.1547	2761.4783	6951
M+H+2Na+1	13.241	936.4933	2761.4913	9925
M+H+2Na+2	13.244	936.8254	2761.4853	8407
M+H+2Na+3	13.251	937.1567	2761.4769	7074
M+H+2Na+4	13.256	937.4817	2761.4497	3386
M+H+2Na+5	13.242	937.8123	2761.4392	2586

Export features: 4664 / groups: 1167

	RT	mass	abund.	height	#ions
463	7.715	1271.5948	11146	1329	3
464	7.716	1579.7000	1223	188	2
465	7.716	748.3860	18753	1216	4
466	7.718	1970.7935	2788	281	3
467	13.242	2826.4049	11853	662	4
468	13.244	2814.4034	60168	1698	8
469	13.245	2816.4119	8707	680	3
470	13.246	2331.1504	3191	291	3
471	13.247	1407.6968	4377	385	2
472	13.249	2761.4877	4778072	79795	78
473	13.250	2433.2736	3736	399	3
474	13.250	2806.4575	16286	650	7
475	13.252	1123.6411	4246	416	2
476	13.253	2068.0019	14029	665	7
477	13.253	2783.4528	27728	1212	8
478	9.159	677.2975	8801	714	2
479	9.159	2173.8394	3492	427	3
480	9.161	1310.6477	1838172	70500	23
481	9.161		12218	1585	1
482	9.162	1334.6137	75374	2869	10
483	10.385	1540.6853	2023	344	2

Raw data → **Background noise removed** → **Individual m/z peaks grouped into isotope clusters** → **Isotope clusters grouped into molecular features**

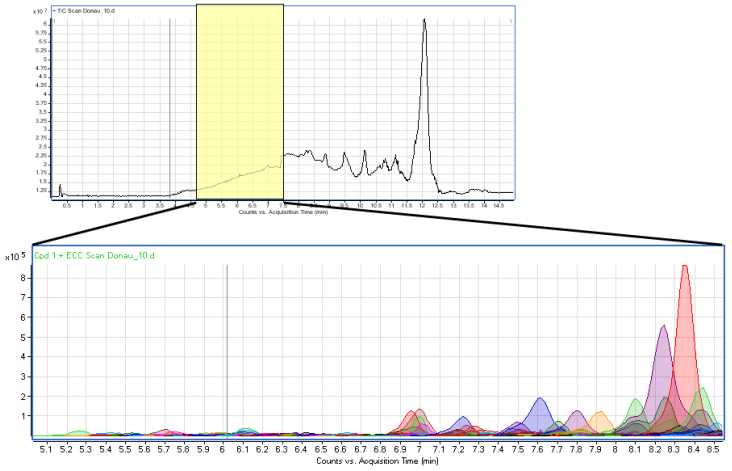
Identification, Quantification, Differential Analysis are performed on *chemically qualified compound data*

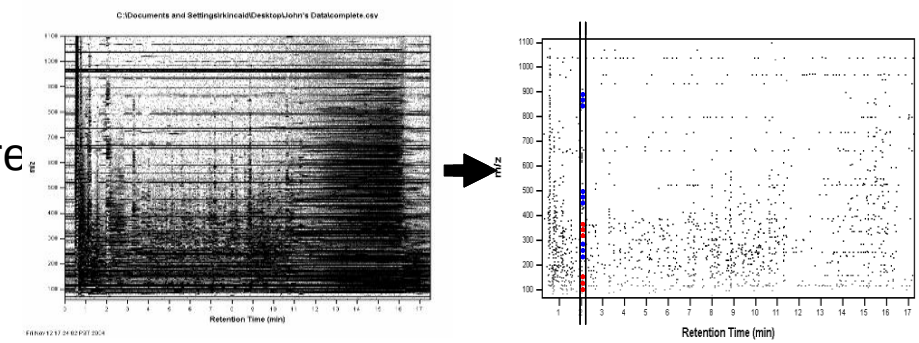
Molecular Feature Extraction (MFE)

Compound Finding Algorithm

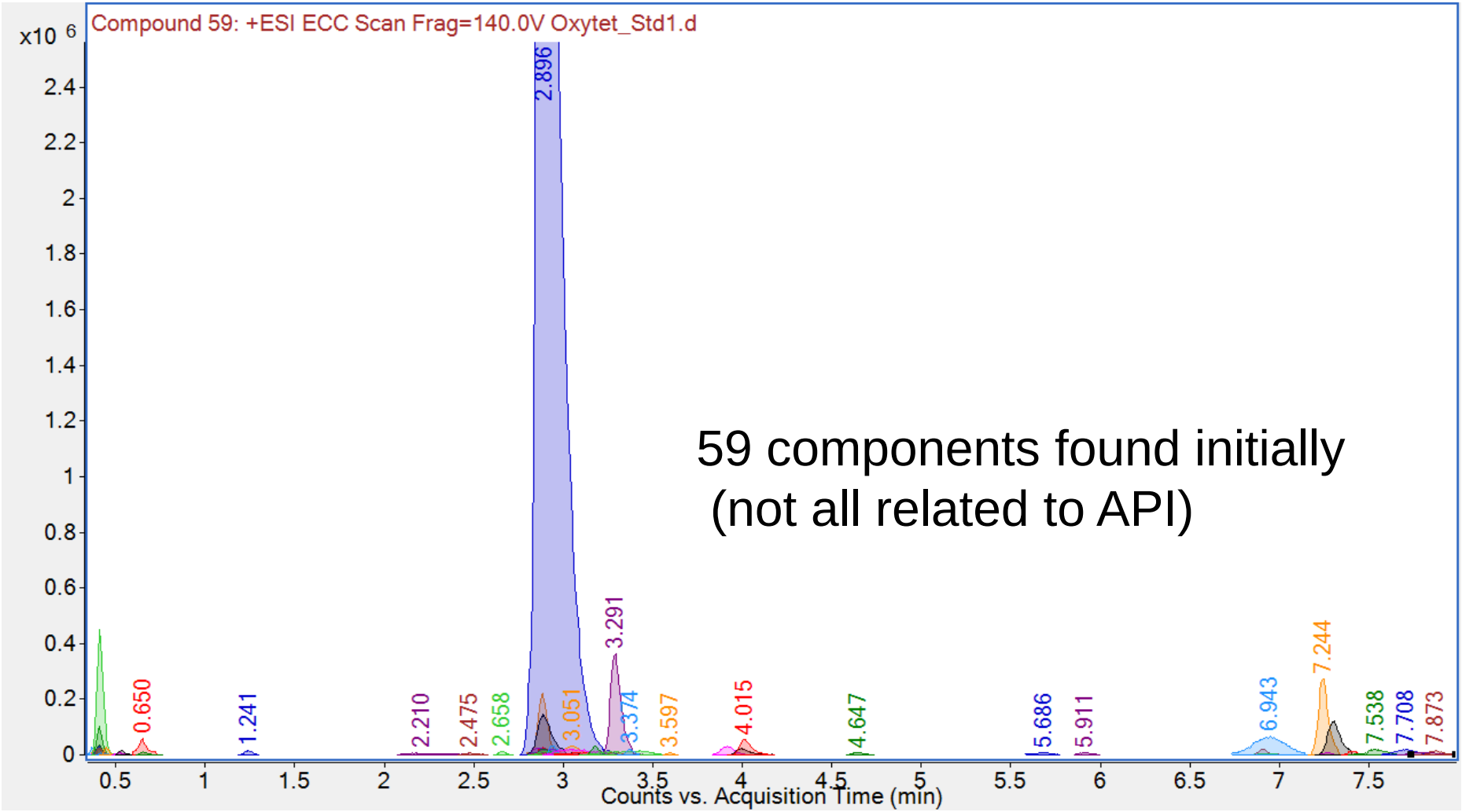
Data is processed by a proprietary feature-finding algorithm

- Finds chromatographic peaks
  - Finds all ions that are related
  - Includes any adducts, such as Na^+ or K^+
  - Includes isotopes A+1, A+2 for all adducts found…
  - Includes different charge states
  - Checks for dimers
  - Creates a compound spectra (ECC)
- Sums all ion signals into one value (Feature with associated Volume)
- Fully automated processing
- Creates data file for export to MassHunter



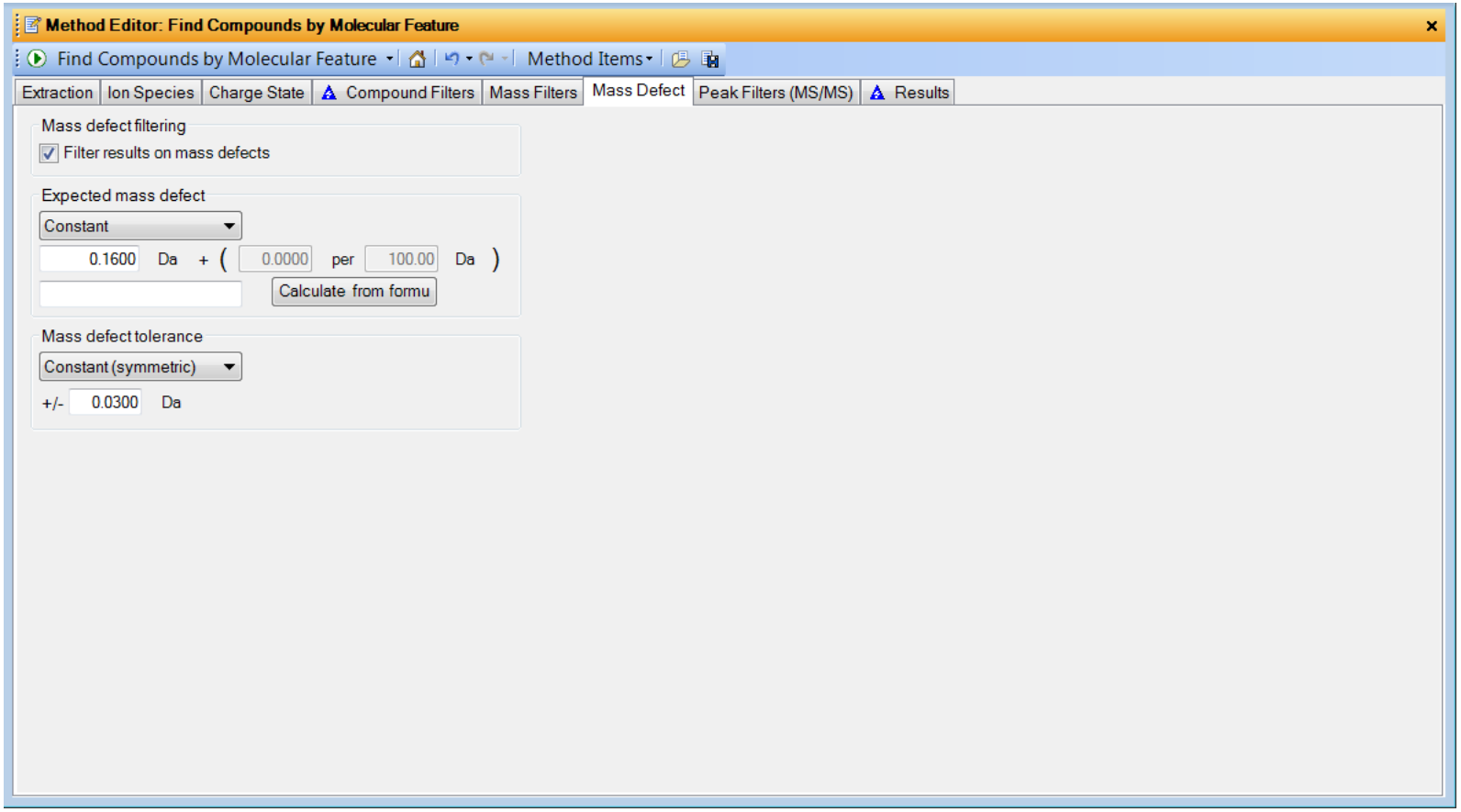


Molecular Feature Extracted (MFE) Component Chromatograms for Oxytetracycline Standard:



59 components found initially
(not all related to API)

Several filters are available to find API relevant impurities (Mass Defect, Common MS/MS Fragment, and MSMS Neutral Loss):



Variation from Nominal Mass for Selected Elements

H = 1.0078

H+ = 1.0073

O = 15.9949

Br = 78.9183

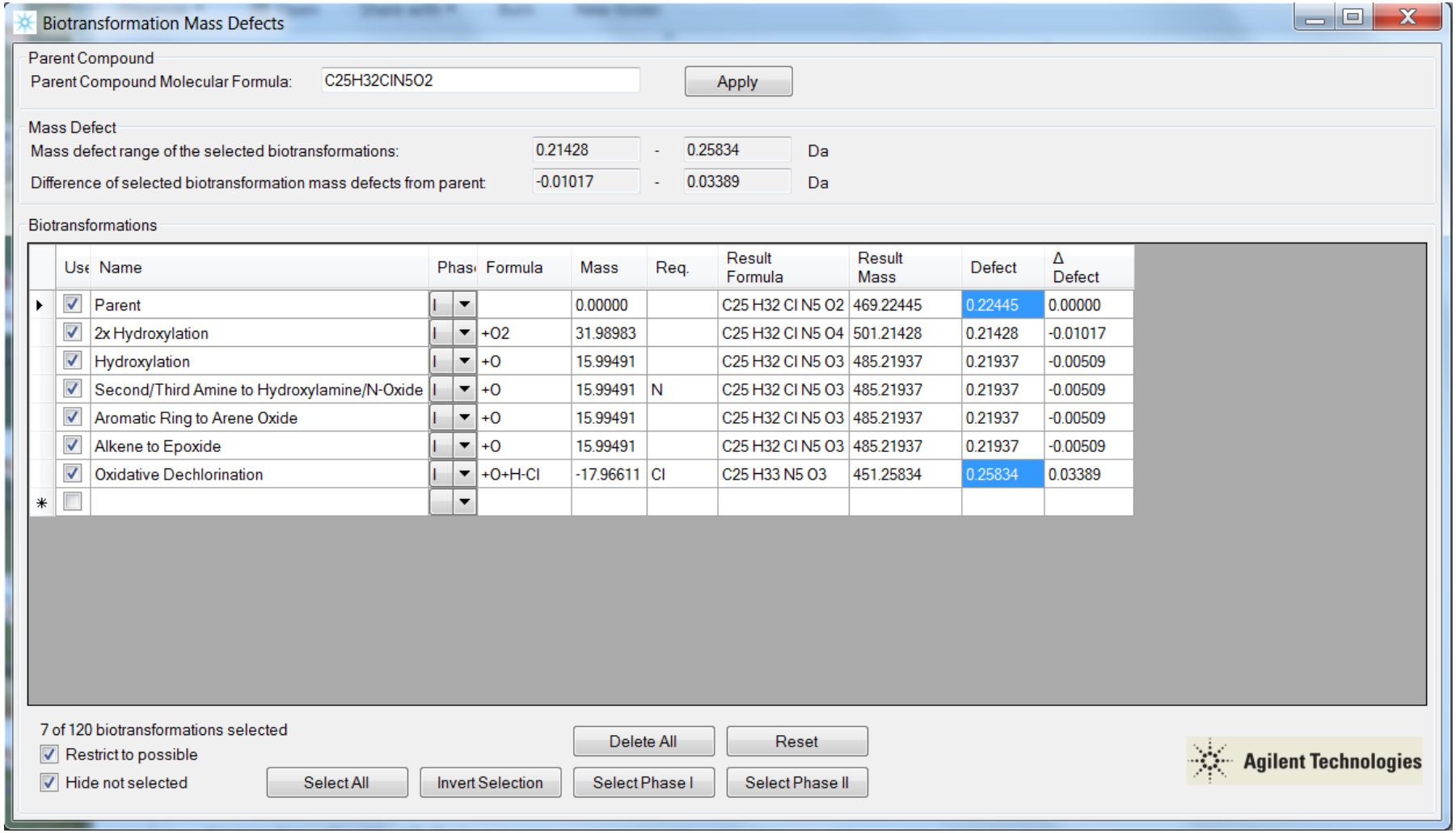
Cl = 34.9689

C12 H18 N O Br = 271.0572

Si O3 C13 H28 = 280.1808

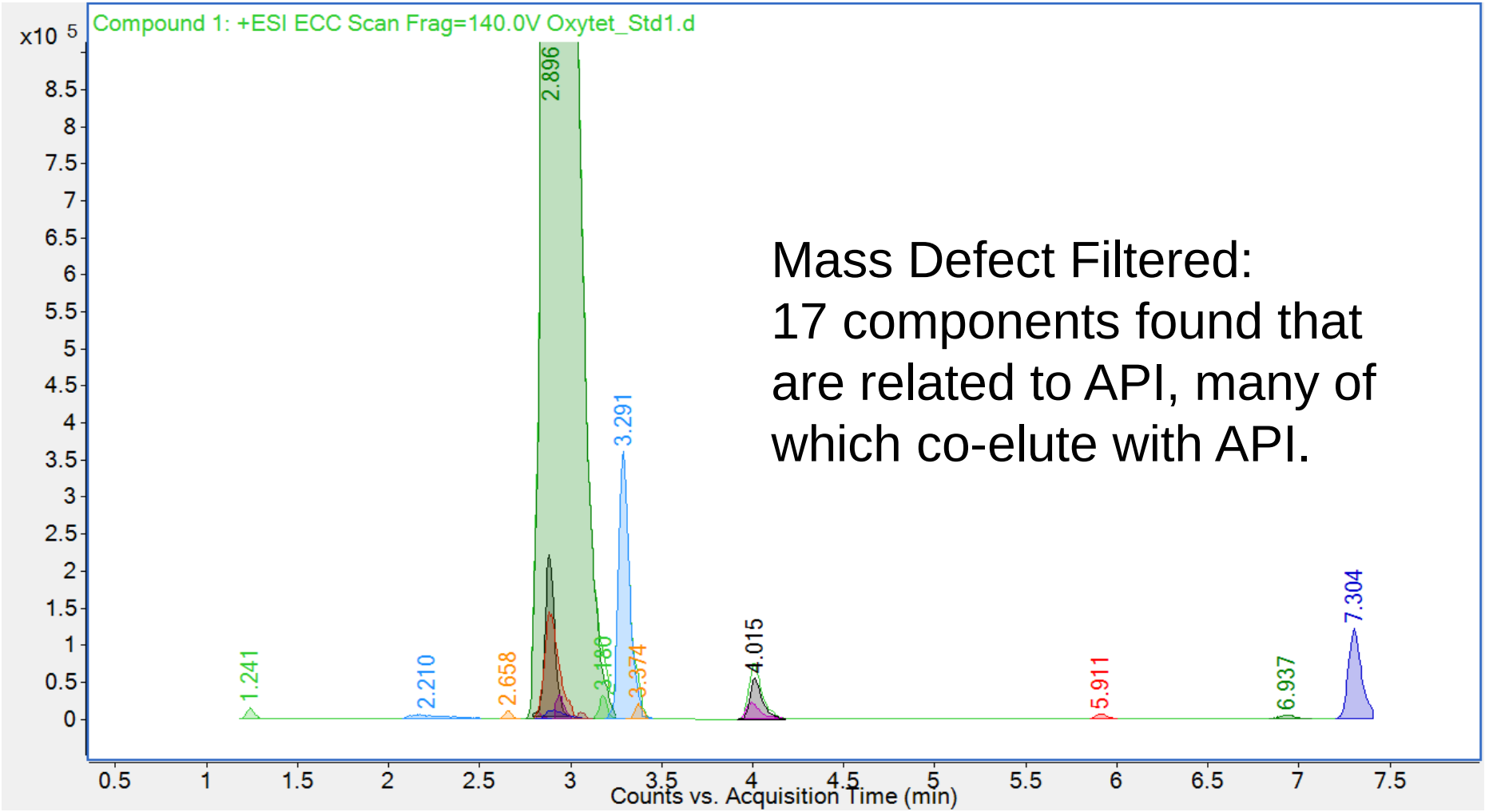
F = 18.9984

How Mass Defect Allows Discovery of Related Components



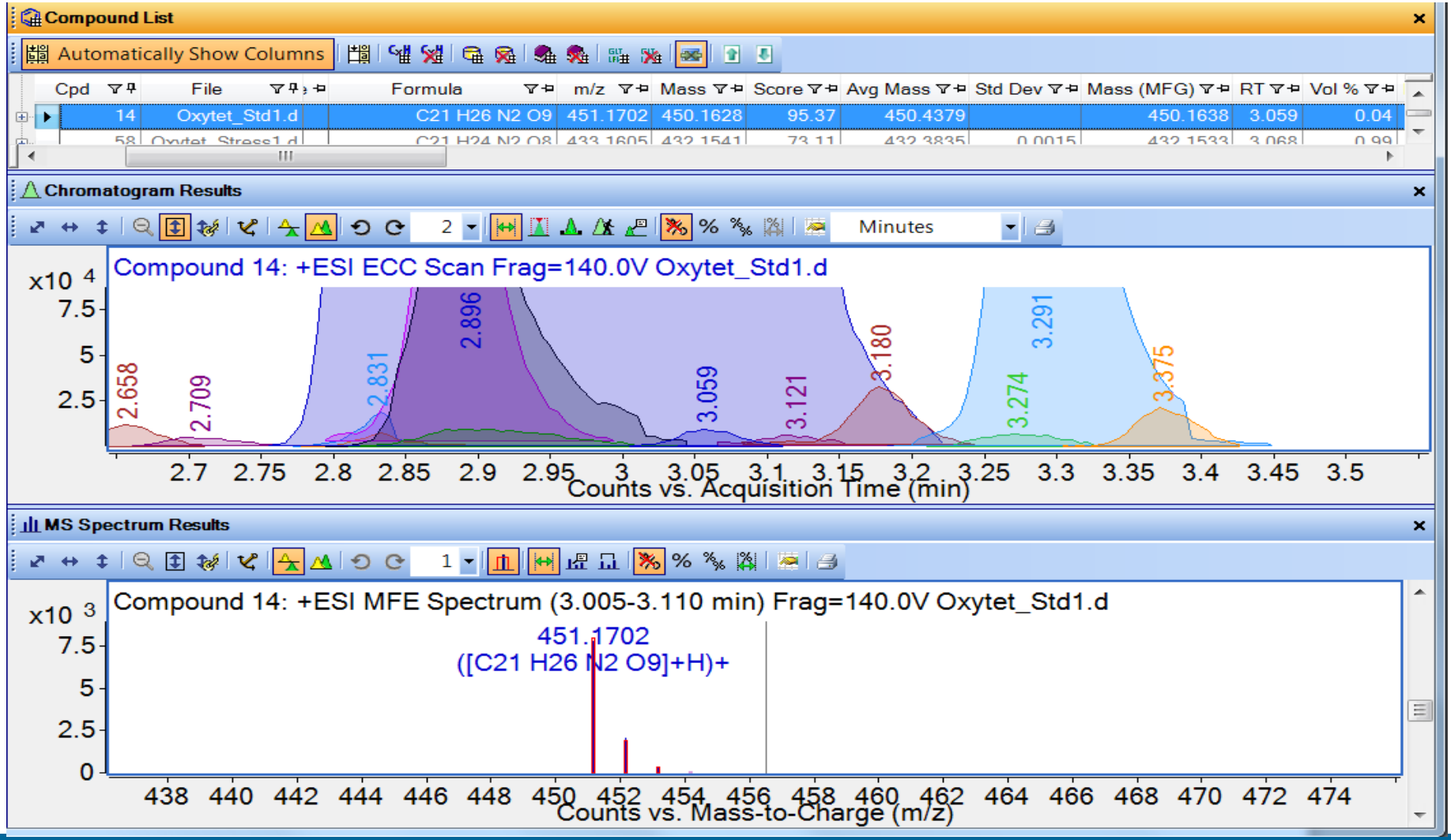
2nd Step – Use Mass Defect Filter to eliminate compounds not related to structure/formula of API

Molecular Feature Extracted Component Chromatograms for Oxytetracycline Standard

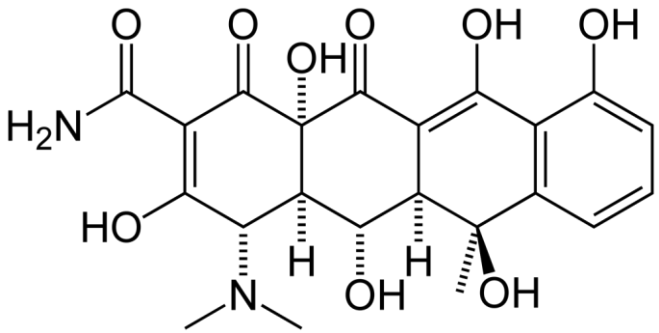


Mass Defect Filtered:
17 components found that are related to API, many of which co-elute with API.

Note impurities hidden under API UV signal, all found by MassHunter MFE:



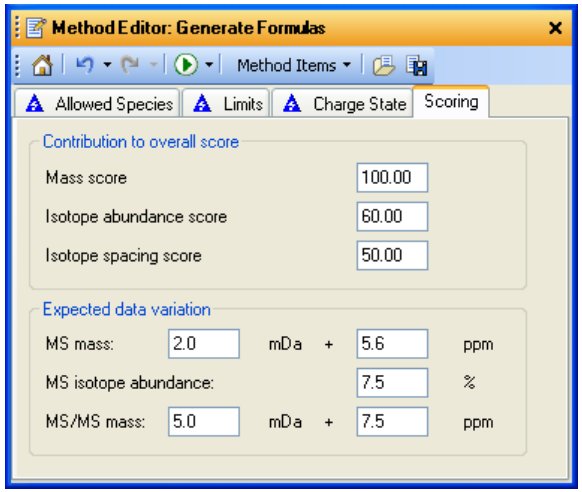
3rd Step – Use Molecular Formula Generator (MFG) to propose elemental formula for all the compounds (“features”) related to API

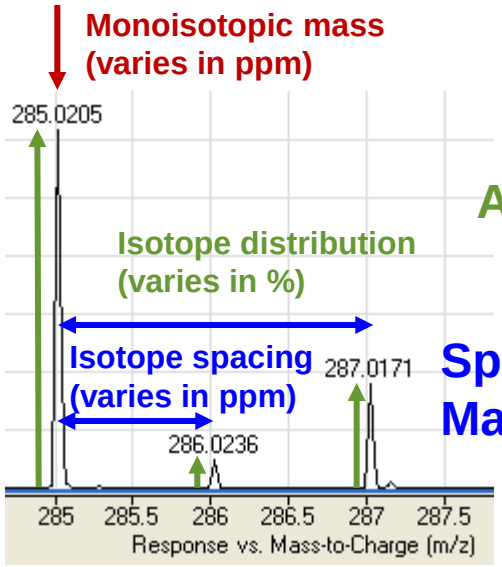


Molecular Formula Generation (MFG) Scoring

Use All Available Information – Consolidated Format

Scoring based on





Mass Match +

Abund. Match +

Spacing Match =

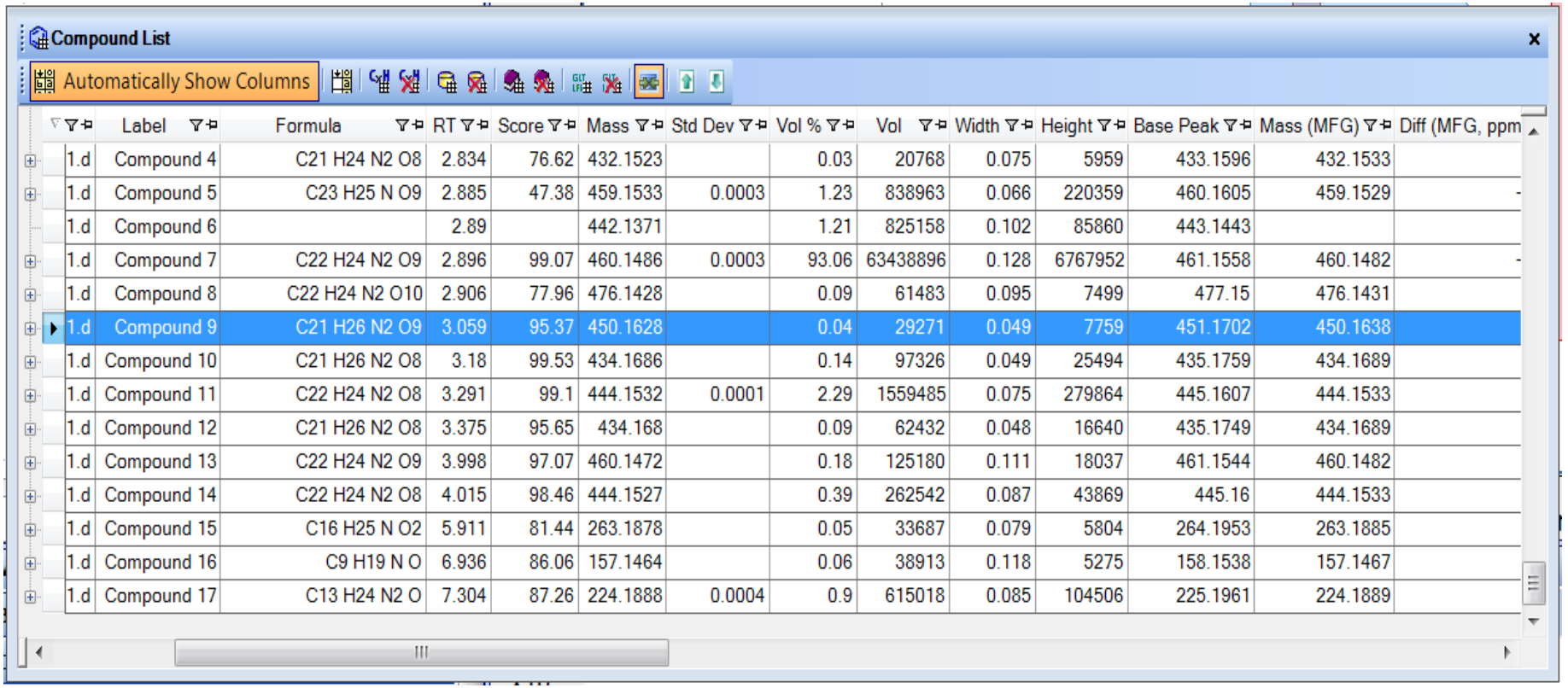
Overall Score

MS Formula Results: Cpd 2: C10H9ClN4O2S

m/z	Ion	Formula	Abundance
285.021	(M+H)+	C10 H10 N4 O2 S	24506.1

Best	Formula (M)	Calc m/z	Score	Cross Score	Mass	Calc Mass	Diff (ppm)	Abs Diff (p	Spacing Matc	Abund Matc	Mass Match	m/z	DBE
✓	C10 H9 Cl N4 O2 S	285.0208	99.55		284.0137	284.0135	-0.71	0.71	99.19	99.26	99.69	285.021	8
	C7 H12 N2 O6 S2	285.021	77.28		284.0137	284.0137	0.01	0.01	99.54	1.93	100	285.021	3
	C7 H13 Cl N4 O2 S2	285.0241	75.57		284.0137	284.0168	11.12	11.12	99.87	83.87	46.22	285.021	3

Mass Defect Filtering yields high proportion of API related components.

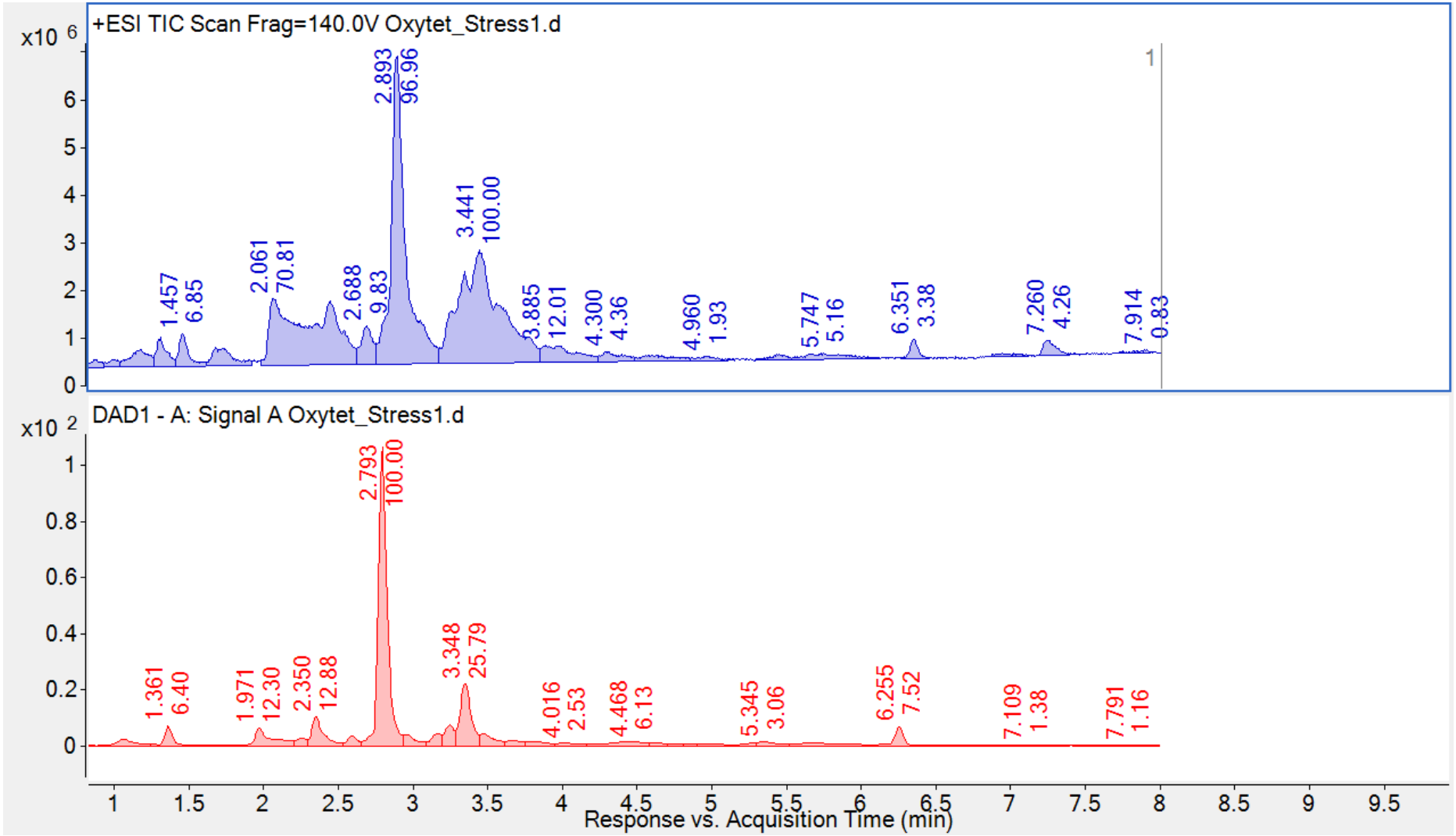


Compound List

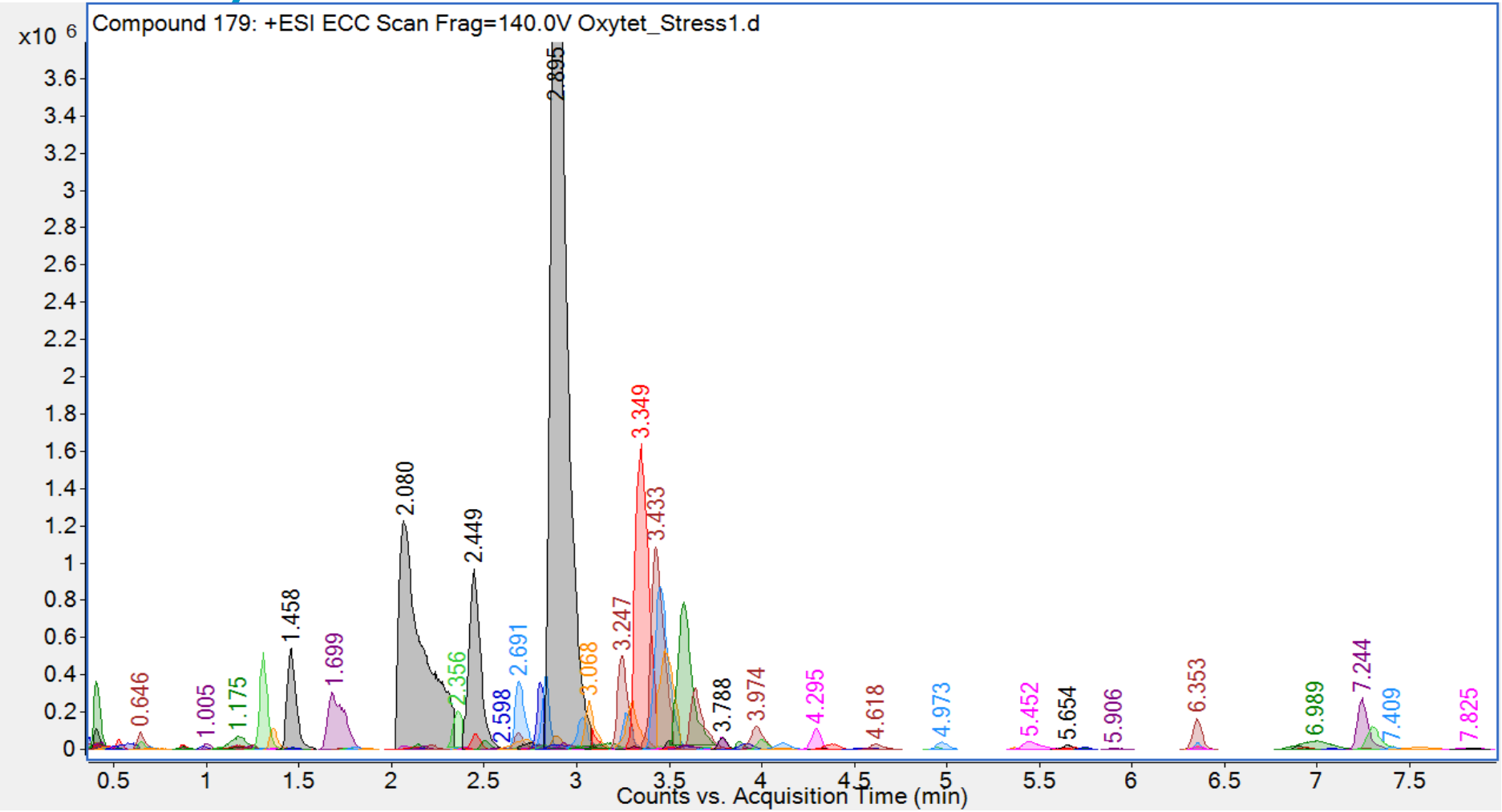
Automatically Show Columns

	Label	Formula	RT	Score	Mass	Std Dev	Vol %	Vol	Width	Height	Base Peak	Mass (MFG)	Diff (MFG, ppm
1.d	Compound 4	C21 H24 N2 O8	2.834	76.62	432.1523		0.03	20768	0.075	5959	433.1596	432.1533	
1.d	Compound 5	C23 H25 N O9	2.885	47.38	459.1533	0.0003	1.23	838963	0.066	220359	460.1605	459.1529	-
1.d	Compound 6		2.89		442.1371		1.21	825158	0.102	85860	443.1443		
1.d	Compound 7	C22 H24 N2 O9	2.896	99.07	460.1486	0.0003	93.06	63438896	0.128	6767952	461.1558	460.1482	-
1.d	Compound 8	C22 H24 N2 O10	2.906	77.96	476.1428		0.09	61483	0.095	7499	477.15	476.1431	
1.d	Compound 9	C21 H26 N2 O9	3.059	95.37	450.1628		0.04	29271	0.049	7759	451.1702	450.1638	
1.d	Compound 10	C21 H26 N2 O8	3.18	99.53	434.1686		0.14	97326	0.049	25494	435.1759	434.1689	
1.d	Compound 11	C22 H24 N2 O8	3.291	99.1	444.1532	0.0001	2.29	1559485	0.075	279864	445.1607	444.1533	
1.d	Compound 12	C21 H26 N2 O8	3.375	95.65	434.168		0.09	62432	0.048	16640	435.1749	434.1689	
1.d	Compound 13	C22 H24 N2 O9	3.998	97.07	460.1472		0.18	125180	0.111	18037	461.1544	460.1482	
1.d	Compound 14	C22 H24 N2 O8	4.015	98.46	444.1527		0.39	262542	0.087	43869	445.16	444.1533	
1.d	Compound 15	C16 H25 N O2	5.911	81.44	263.1878		0.05	33687	0.079	5804	264.1953	263.1885	
1.d	Compound 16	C9 H19 N O	6.936	86.06	157.1464		0.06	38913	0.118	5275	158.1538	157.1467	
1.d	Compound 17	C13 H24 N2 O	7.304	87.26	224.1888	0.0004	0.9	615018	0.085	104506	225.1961	224.1889	

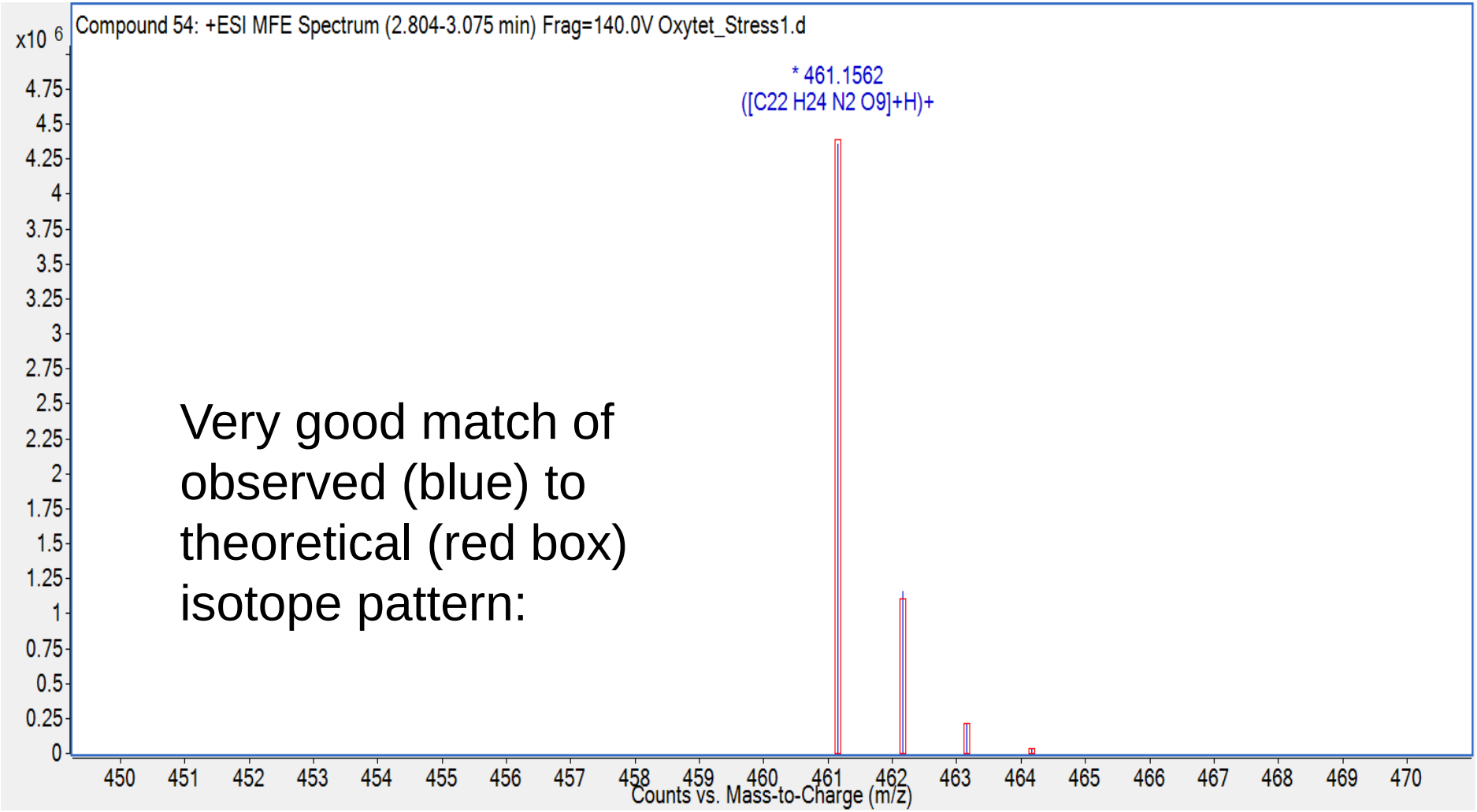
TIC and UV Chromatograms of H2O Stressed Oxytetracycline



Molecular Feature Extracted and Mass Defect Filtered Component Chromatograms for Oxytetracycline (H2O Stressed)

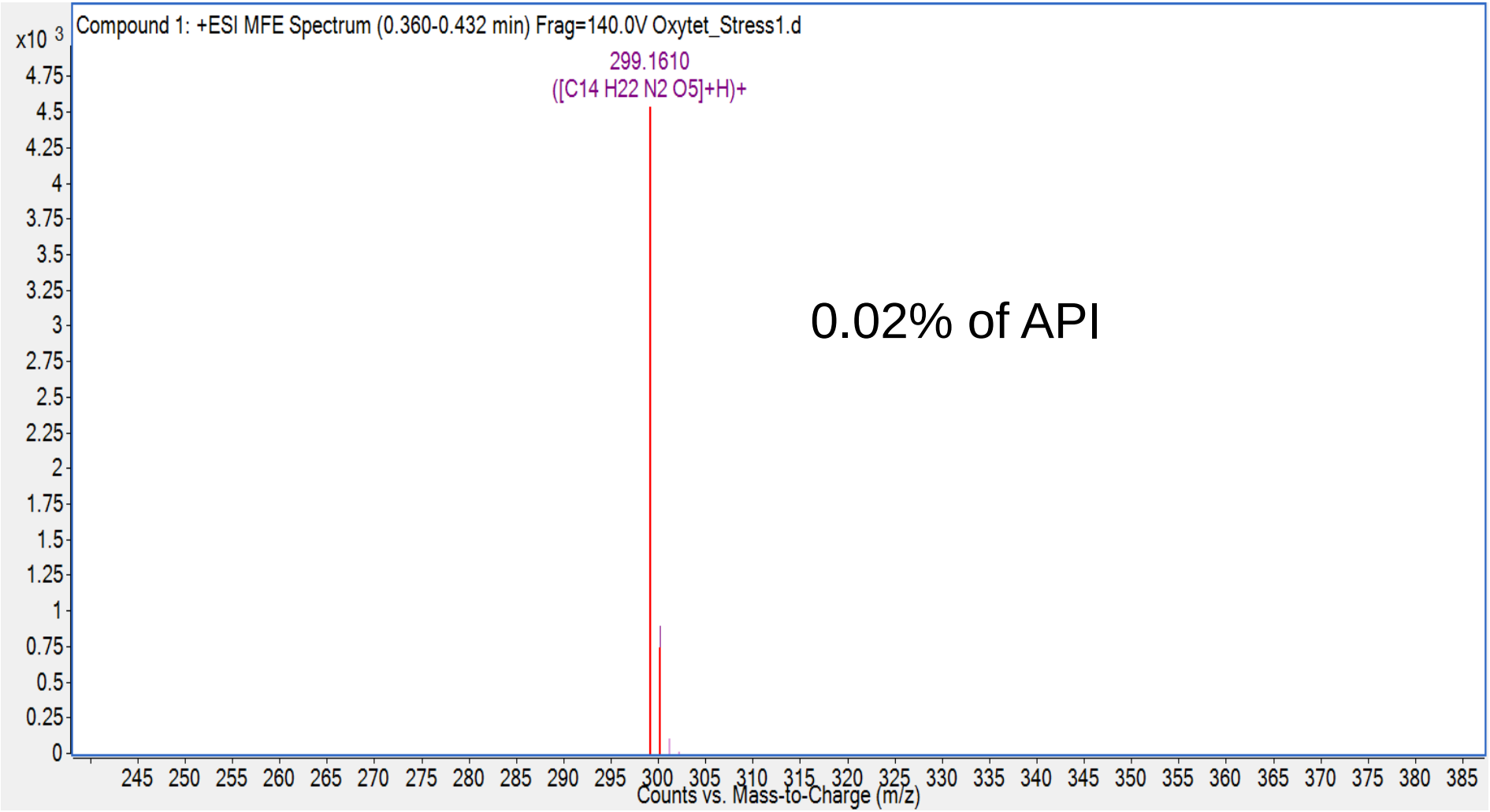


Dynamic Range for API (oxytetracycline) in Stressed Sample: Area (V) = 32,008,508

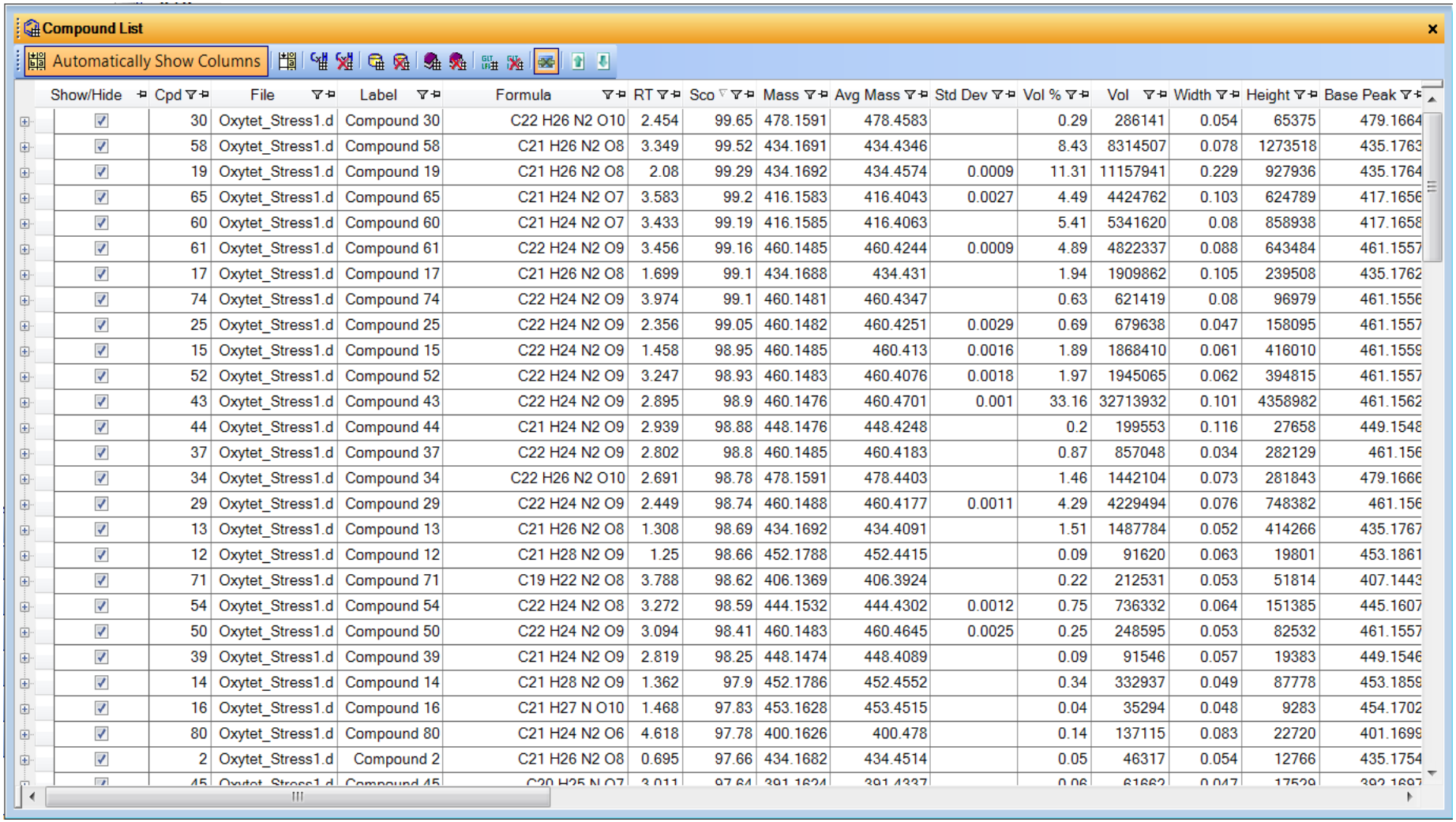


Very good match of observed (blue) to theoretical (red box) isotope pattern:

Dynamic Range for Degradation Component at 0.02% level in Stressed Sample: Area (V) = 7,057



Formula Generation (MFG) shows the efficiency of extracting related components from H2O stressed sample:



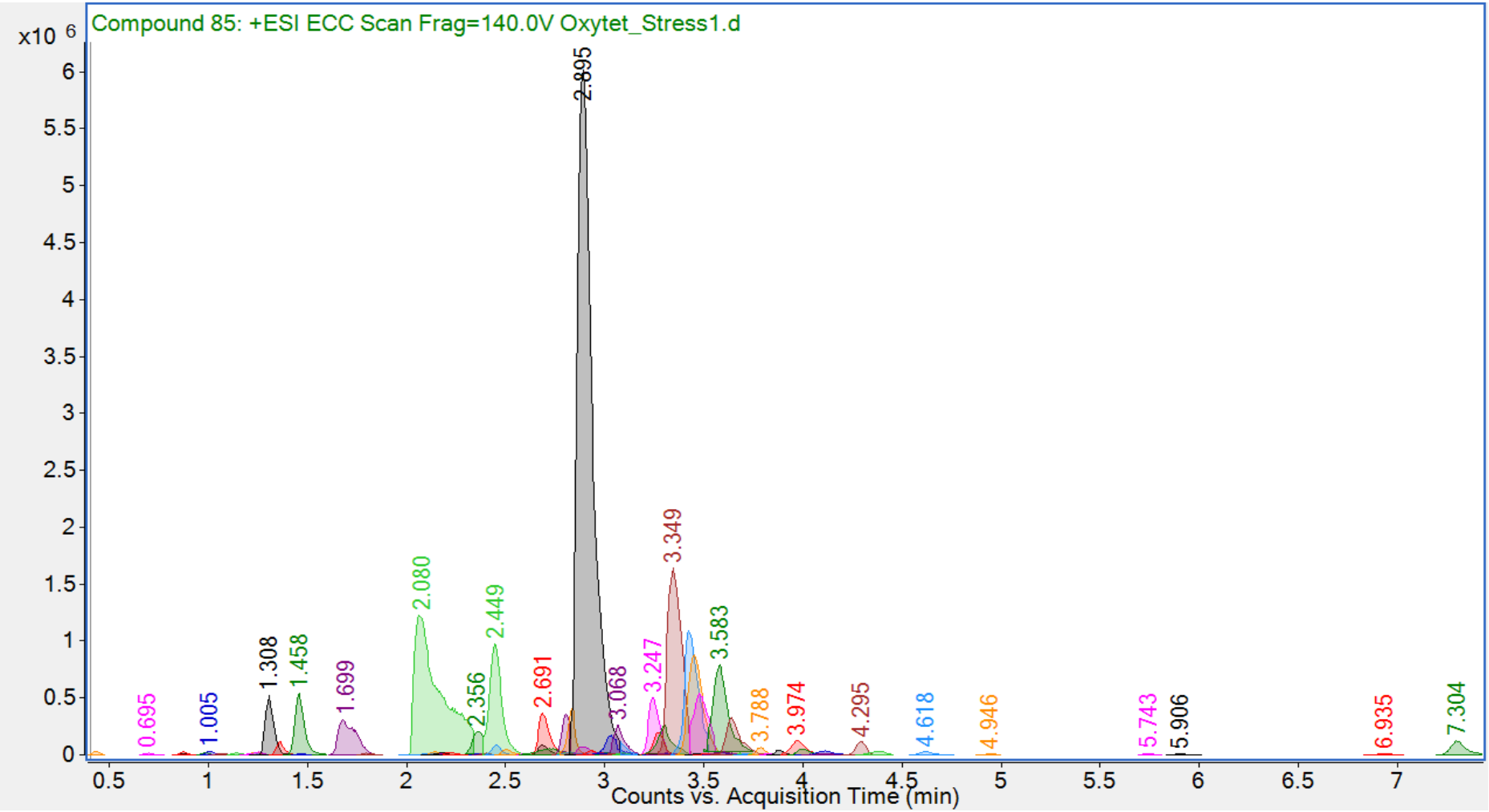
Compound List

Automatically Show Columns

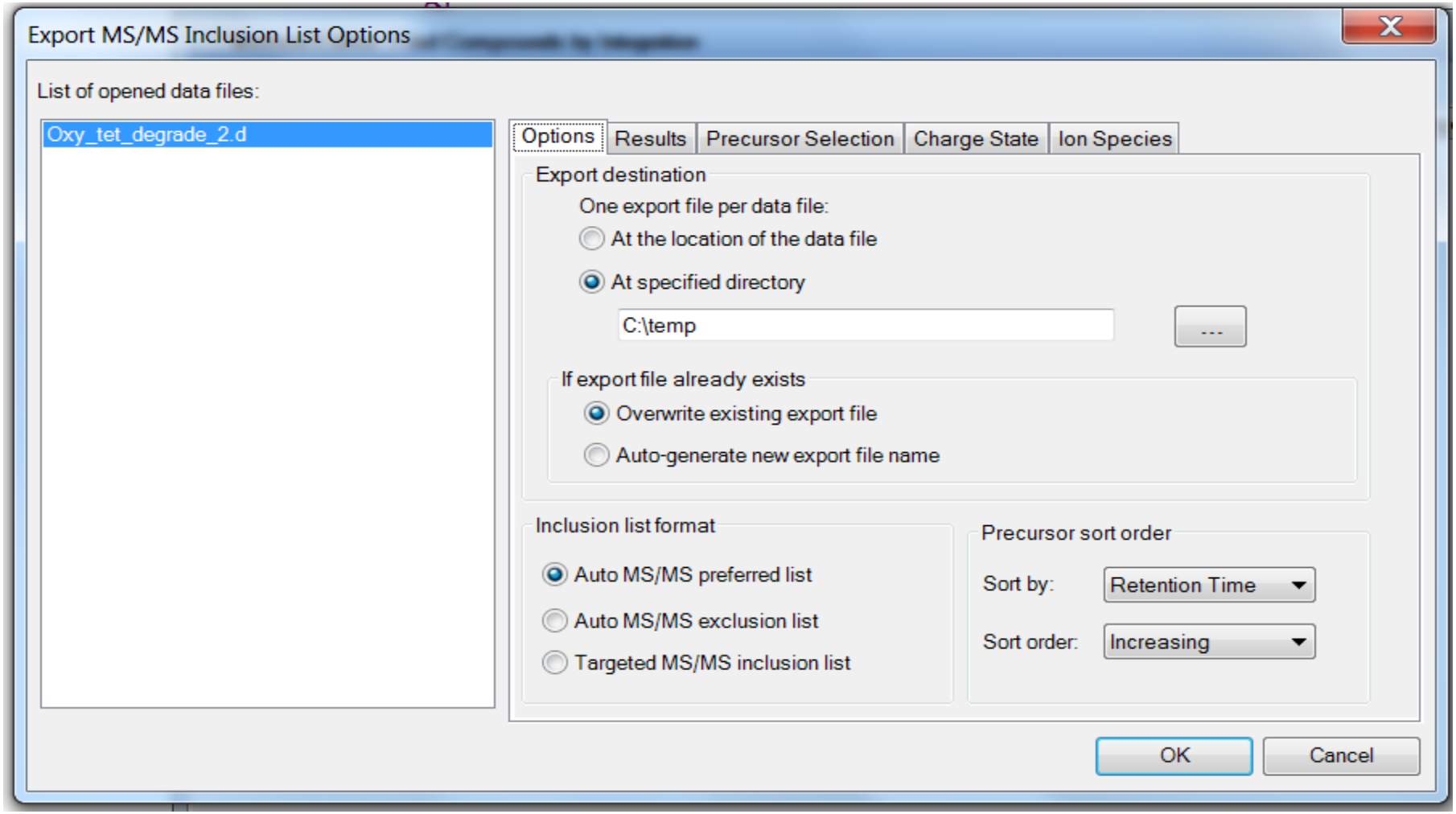
Show/Hide	Cpd	File	Label	Formula	RT	Sco	Mass	Avg Mass	Std Dev	Vol %	Vol	Width	Height	Base Peak
☑	30	Oxytet_Stress1.d	Compound 30	C22 H26 N2 O10	2.454	99.65	478.1591	478.4583		0.29	286141	0.054	65375	479.1664
☑	58	Oxytet_Stress1.d	Compound 58	C21 H26 N2 O8	3.349	99.52	434.1691	434.4346		8.43	8314507	0.078	1273518	435.1763
☑	19	Oxytet_Stress1.d	Compound 19	C21 H26 N2 O8	2.08	99.29	434.1692	434.4574	0.0009	11.31	11157941	0.229	927936	435.1764
☑	65	Oxytet_Stress1.d	Compound 65	C21 H24 N2 O7	3.583	99.2	416.1583	416.4043	0.0027	4.49	4424762	0.103	624789	417.1656
☑	60	Oxytet_Stress1.d	Compound 60	C21 H24 N2 O7	3.433	99.19	416.1585	416.4063		5.41	5341620	0.08	858938	417.1658
☑	61	Oxytet_Stress1.d	Compound 61	C22 H24 N2 O9	3.456	99.16	460.1485	460.4244	0.0009	4.89	4822337	0.088	643484	461.1557
☑	17	Oxytet_Stress1.d	Compound 17	C21 H26 N2 O8	1.699	99.1	434.1688	434.431		1.94	1909862	0.105	239508	435.1762
☑	74	Oxytet_Stress1.d	Compound 74	C22 H24 N2 O9	3.974	99.1	460.1481	460.4347		0.63	621419	0.08	96979	461.1556
☑	25	Oxytet_Stress1.d	Compound 25	C22 H24 N2 O9	2.356	99.05	460.1482	460.4251	0.0029	0.69	679638	0.047	158095	461.1557
☑	15	Oxytet_Stress1.d	Compound 15	C22 H24 N2 O9	1.458	98.95	460.1485	460.413	0.0016	1.89	1868410	0.061	416010	461.1559
☑	52	Oxytet_Stress1.d	Compound 52	C22 H24 N2 O9	3.247	98.93	460.1483	460.4076	0.0018	1.97	1945065	0.062	394815	461.1557
☑	43	Oxytet_Stress1.d	Compound 43	C22 H24 N2 O9	2.895	98.9	460.1476	460.4701	0.001	33.16	32713932	0.101	4358982	461.1562
☑	44	Oxytet_Stress1.d	Compound 44	C21 H24 N2 O9	2.939	98.88	448.1476	448.4248		0.2	199553	0.116	27658	449.1548
☑	37	Oxytet_Stress1.d	Compound 37	C22 H24 N2 O9	2.802	98.8	460.1485	460.4183		0.87	857048	0.034	282129	461.156
☑	34	Oxytet_Stress1.d	Compound 34	C22 H26 N2 O10	2.691	98.78	478.1591	478.4403		1.46	1442104	0.073	281843	479.1666
☑	29	Oxytet_Stress1.d	Compound 29	C22 H24 N2 O9	2.449	98.74	460.1488	460.4177	0.0011	4.29	4229494	0.076	748382	461.156
☑	13	Oxytet_Stress1.d	Compound 13	C21 H26 N2 O8	1.308	98.69	434.1692	434.4091		1.51	1487784	0.052	414266	435.1767
☑	12	Oxytet_Stress1.d	Compound 12	C21 H28 N2 O9	1.25	98.66	452.1788	452.4415		0.09	91620	0.063	19801	453.1861
☑	71	Oxytet_Stress1.d	Compound 71	C19 H22 N2 O8	3.788	98.62	406.1369	406.3924		0.22	212531	0.053	51814	407.1443
☑	54	Oxytet_Stress1.d	Compound 54	C22 H24 N2 O8	3.272	98.59	444.1532	444.4302	0.0012	0.75	736332	0.064	151385	445.1607
☑	50	Oxytet_Stress1.d	Compound 50	C22 H24 N2 O9	3.094	98.41	460.1483	460.4645	0.0025	0.25	248595	0.053	82532	461.1557
☑	39	Oxytet_Stress1.d	Compound 39	C21 H24 N2 O9	2.819	98.25	448.1474	448.4089		0.09	91546	0.057	19383	449.1546
☑	14	Oxytet_Stress1.d	Compound 14	C21 H28 N2 O9	1.362	97.9	452.1786	452.4552		0.34	332937	0.049	87778	453.1859
☑	16	Oxytet_Stress1.d	Compound 16	C21 H27 N O10	1.468	97.83	453.1628	453.4515		0.04	35294	0.048	9283	454.1702
☑	80	Oxytet_Stress1.d	Compound 80	C21 H24 N2 O6	4.618	97.78	400.1626	400.478		0.14	137115	0.083	22720	401.1699
☑	2	Oxytet_Stress1.d	Compound 2	C21 H26 N2 O8	0.695	97.66	434.1682	434.4514		0.05	46317	0.054	12766	435.1754
☑	45	Oxytet_Stress1.d	Compound 45	C20 H25 N O7	3.011	97.64	391.1624	391.4337		0.06	61662	0.047	17529	392.1697

4th Step – Now that a target list of impurities has been created, now acquire high-quality MS/MS spectra on just these compounds related to the API.

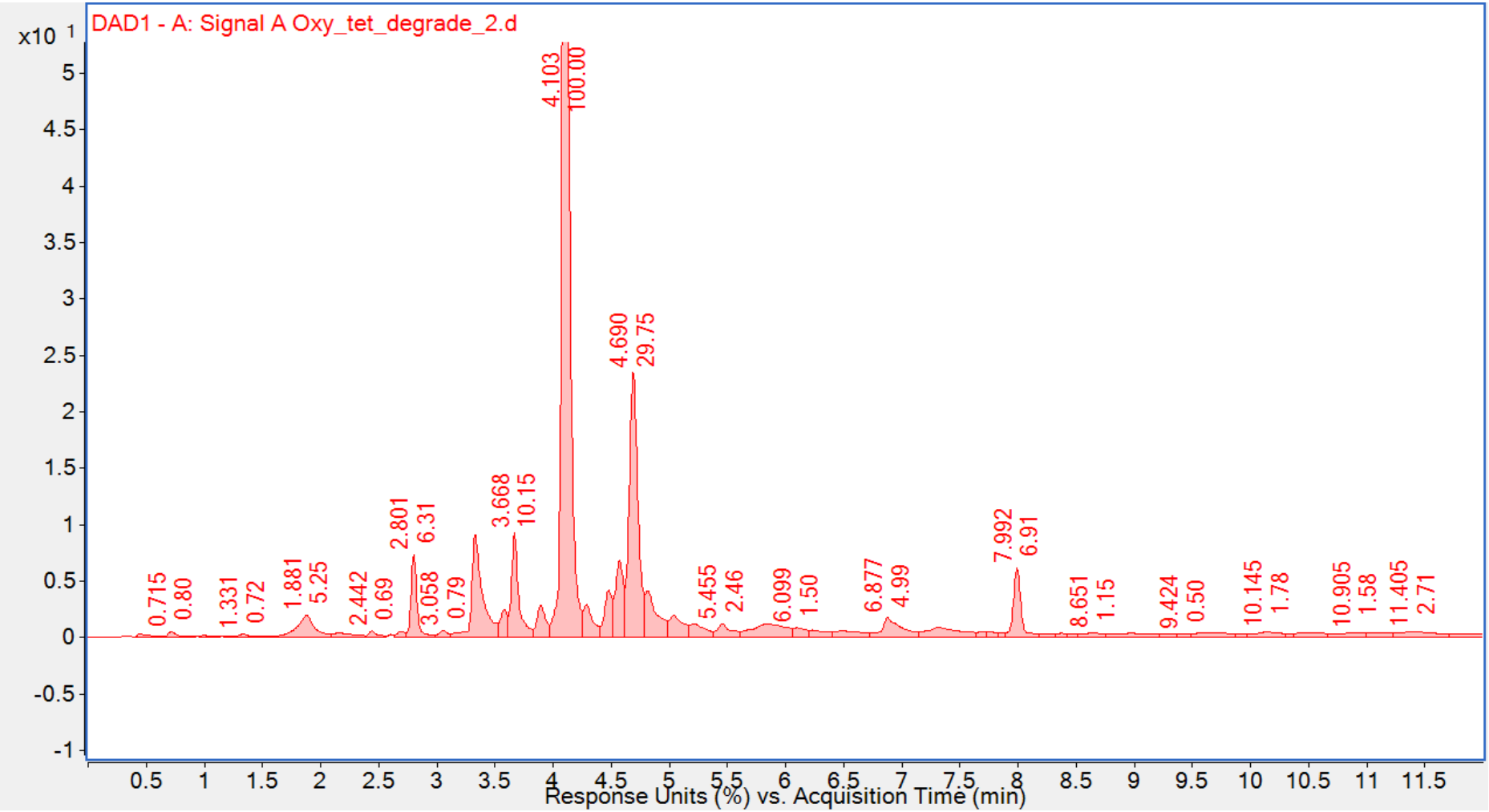
API related components in H2O stressed sample can direct the MS/MS experiments:



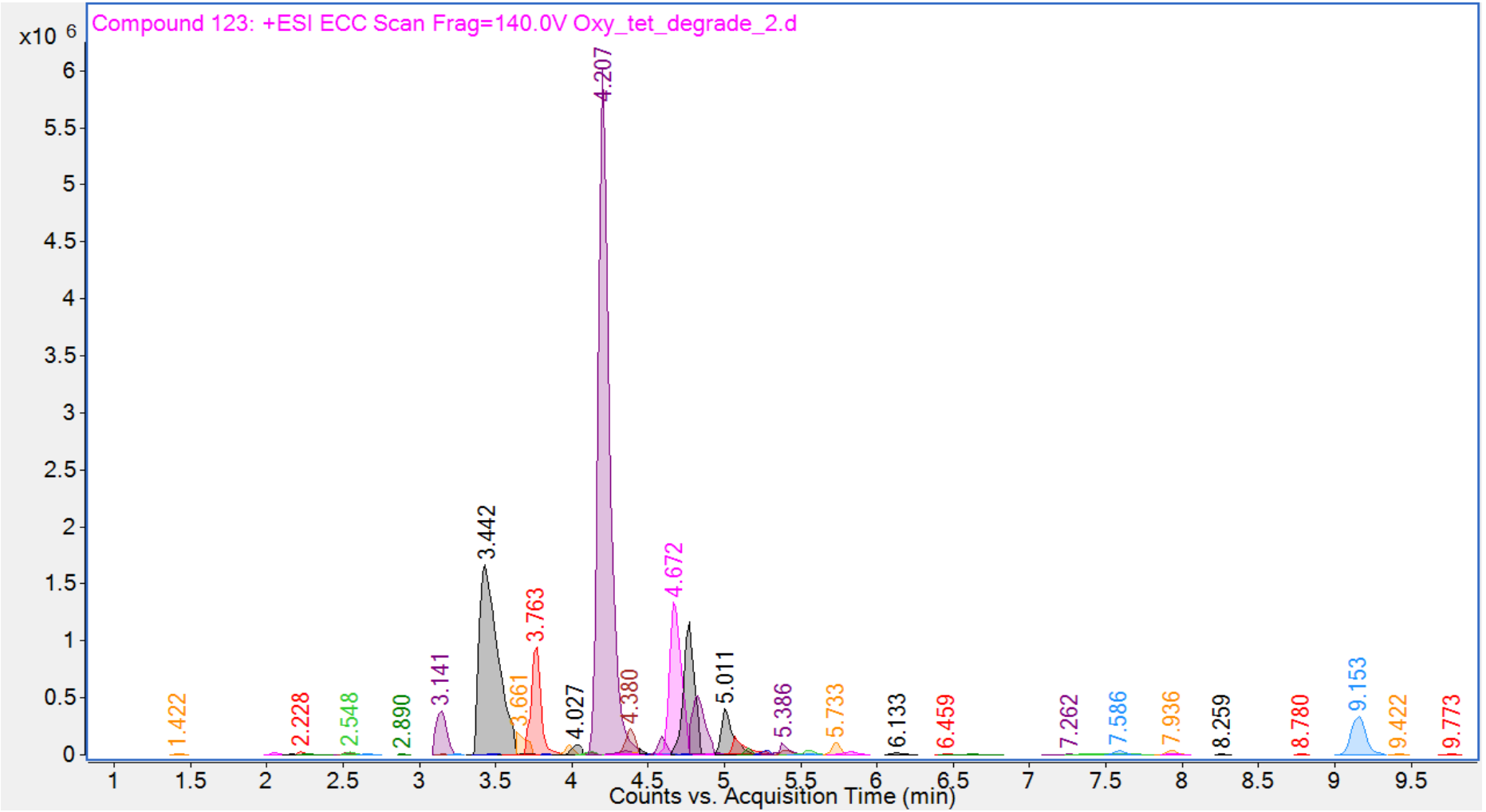
Components are exported automatically by RT and mass for high efficiency MS/MS:



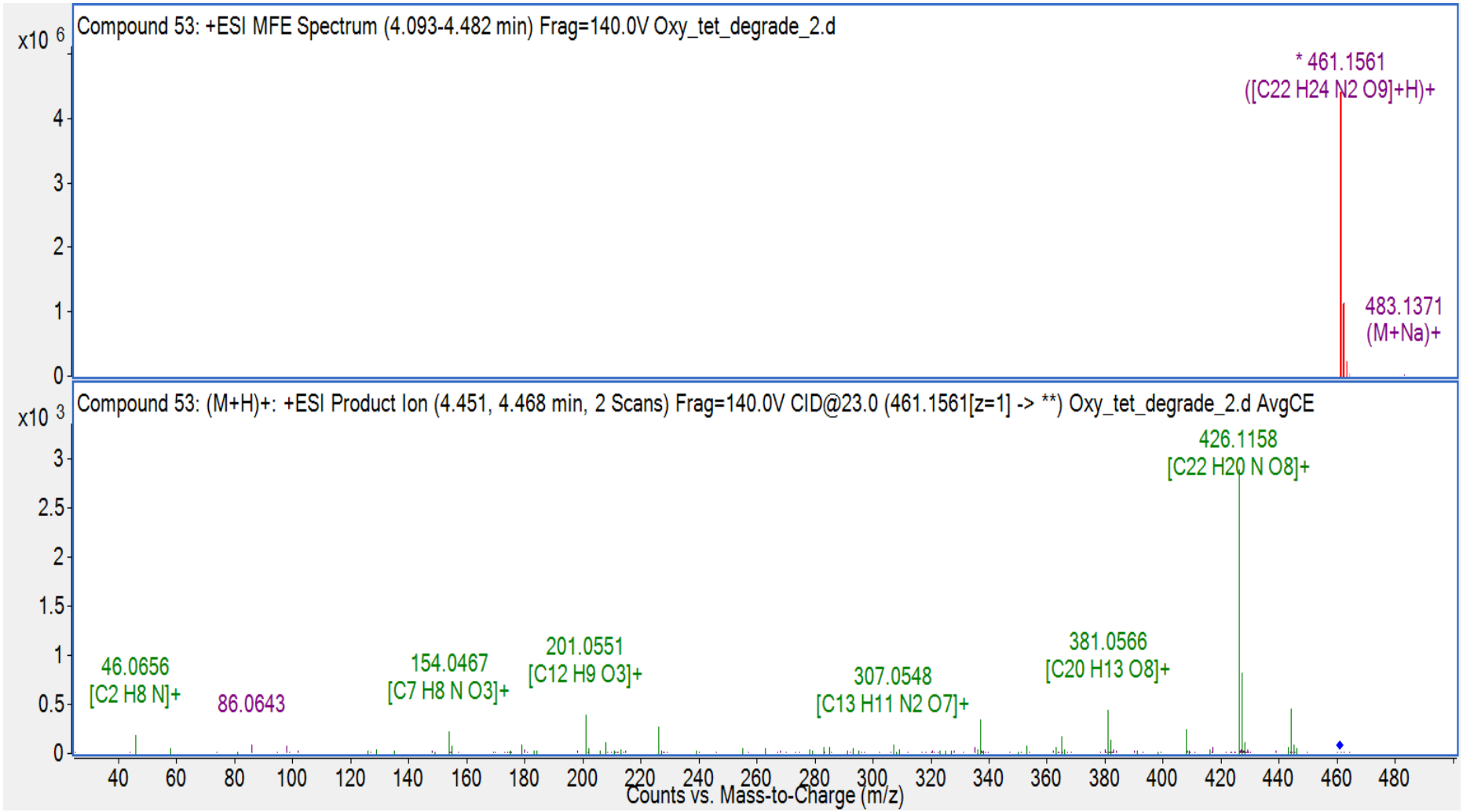
UV Chromatogram for for Oxytetracycline H2O Stressed from Auto MS/MS experiment



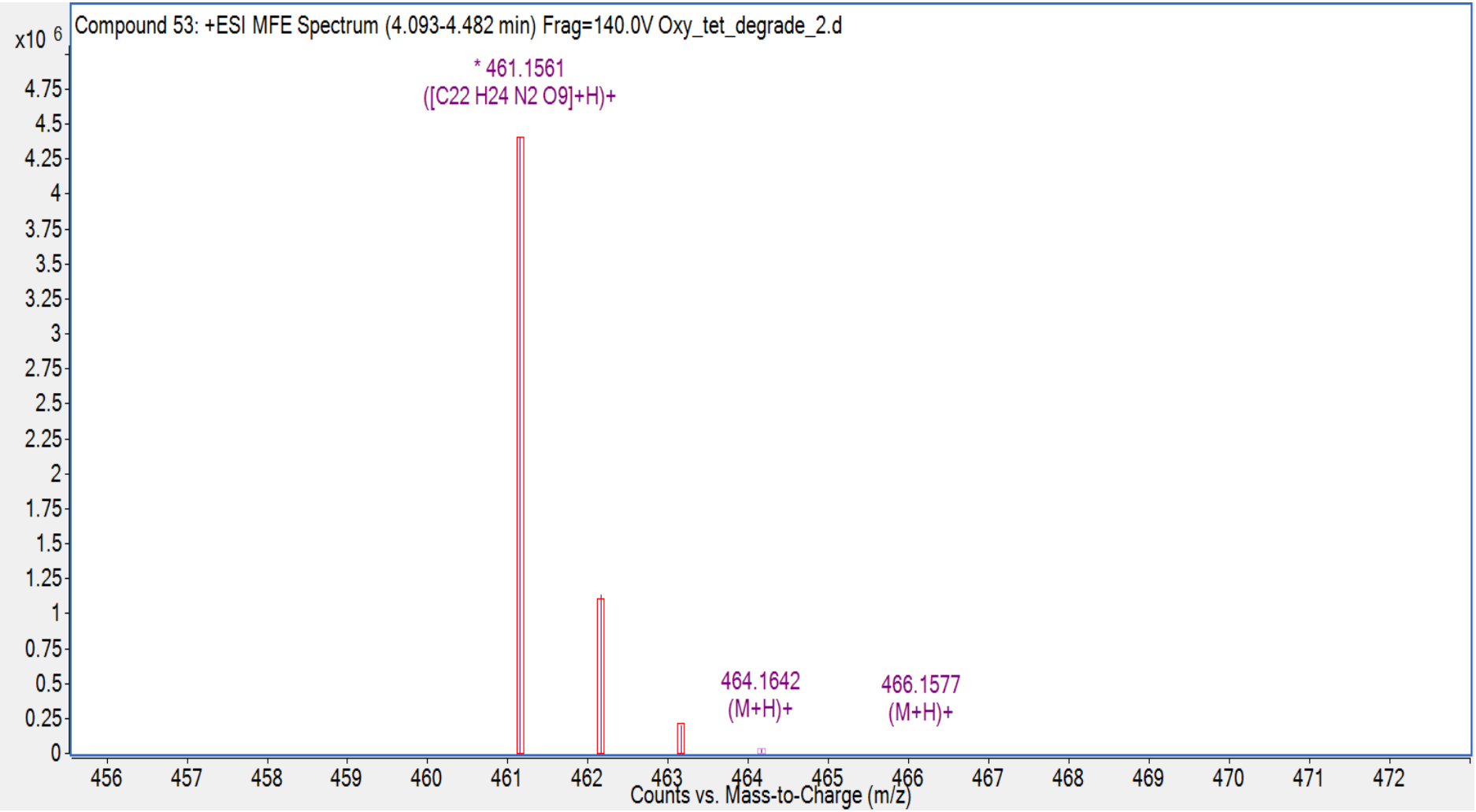
Molecular Feature Extracted Component Chromatograms for Oxytetracycline H2O Stressed from Auto MS/MS experiment



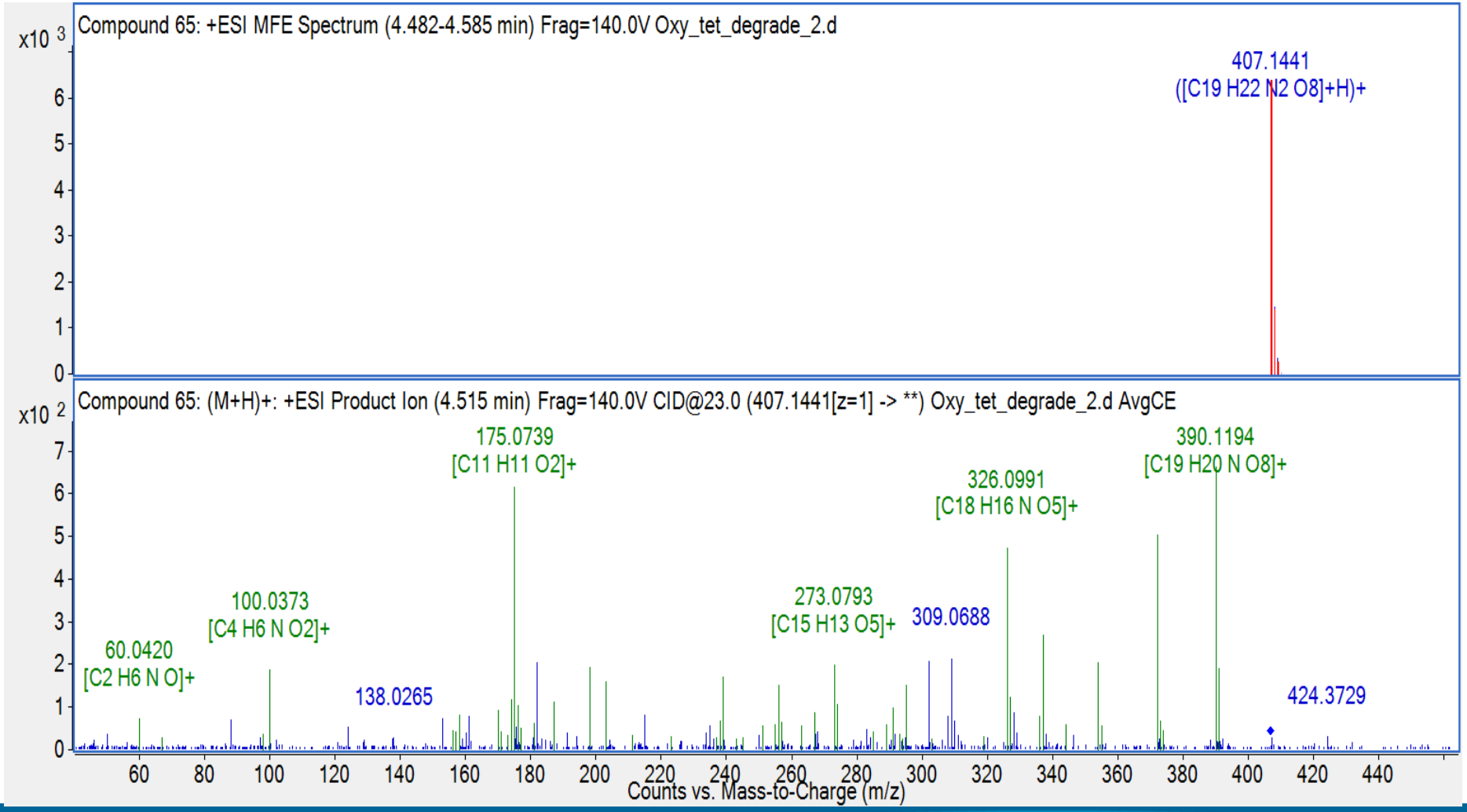
MS/MS data packet for API in H2O stressed sample with formulas assigned for precursor and fragments



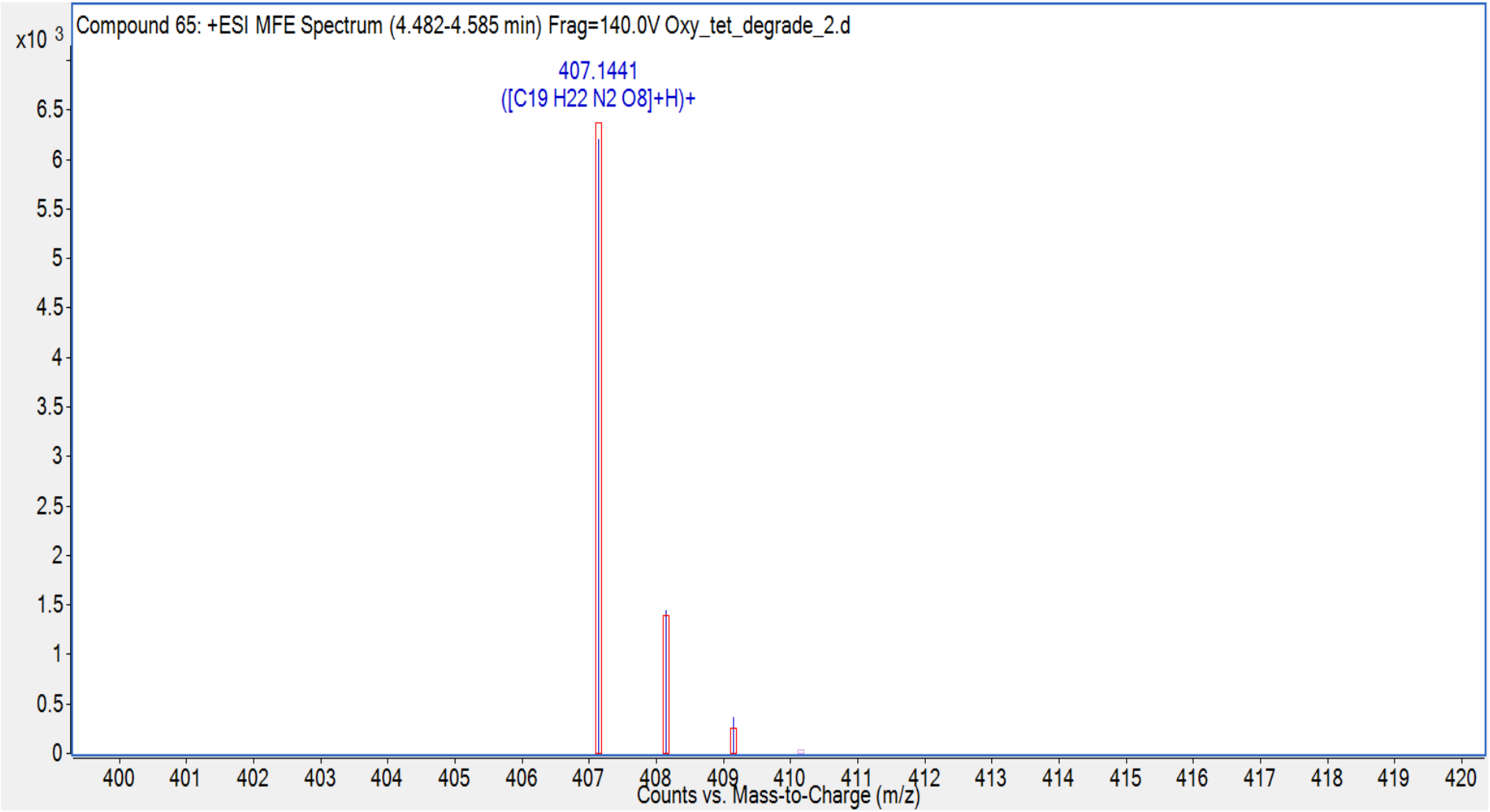
Precursor mass and isotope match for API in H2O stressed sample



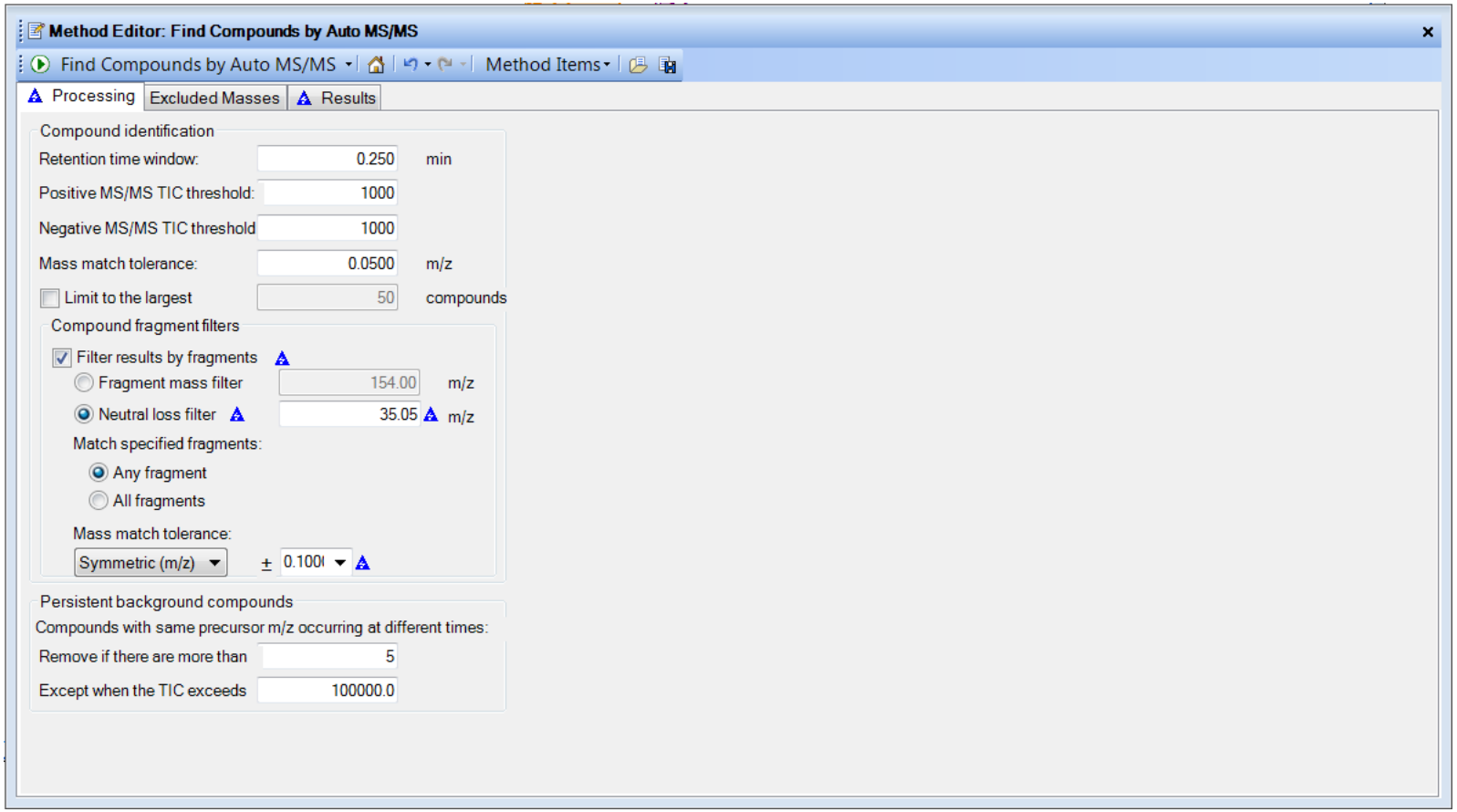
MS/MS data packet for component at 0.03% rel area of API in H2O stressed sample with formulas assigned for precursor and fragments:



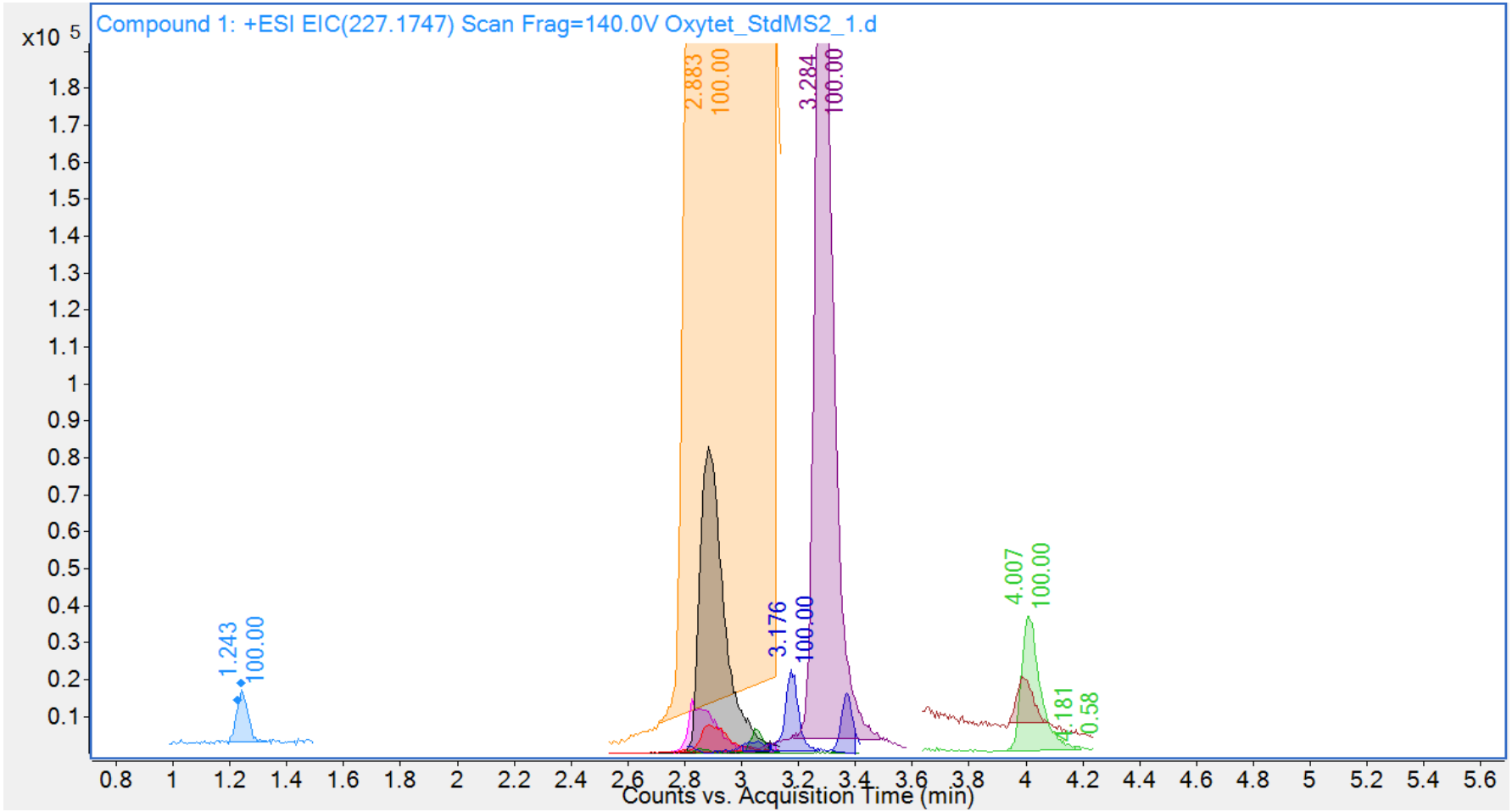
Precursor mass and isotope match for component at 0.03% rel area of API in H2O stressed sample



Find by AutoMSMS components related by common neutral loss and or common fragment ion

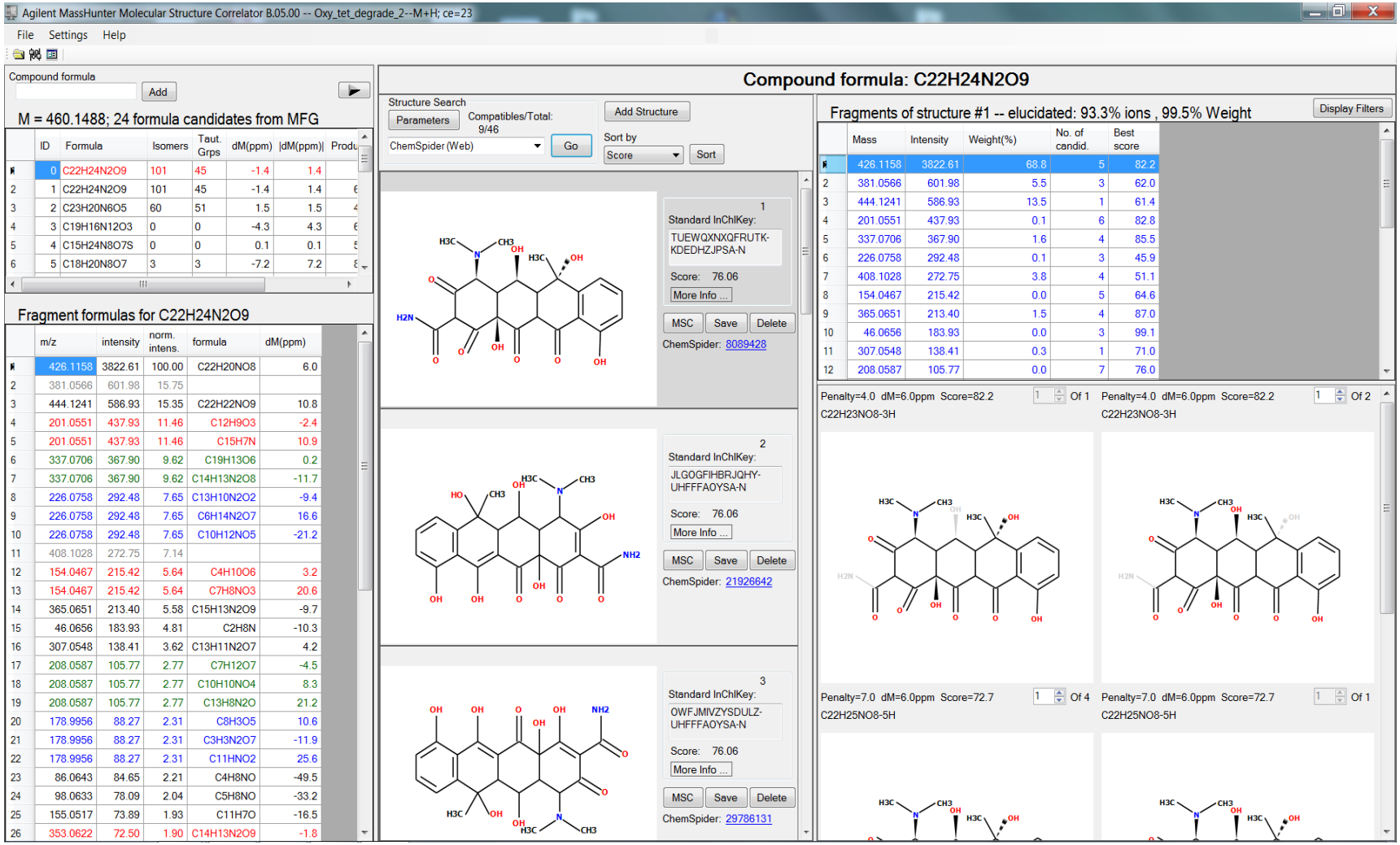


Find by AutoMSMS components related by common neutral loss

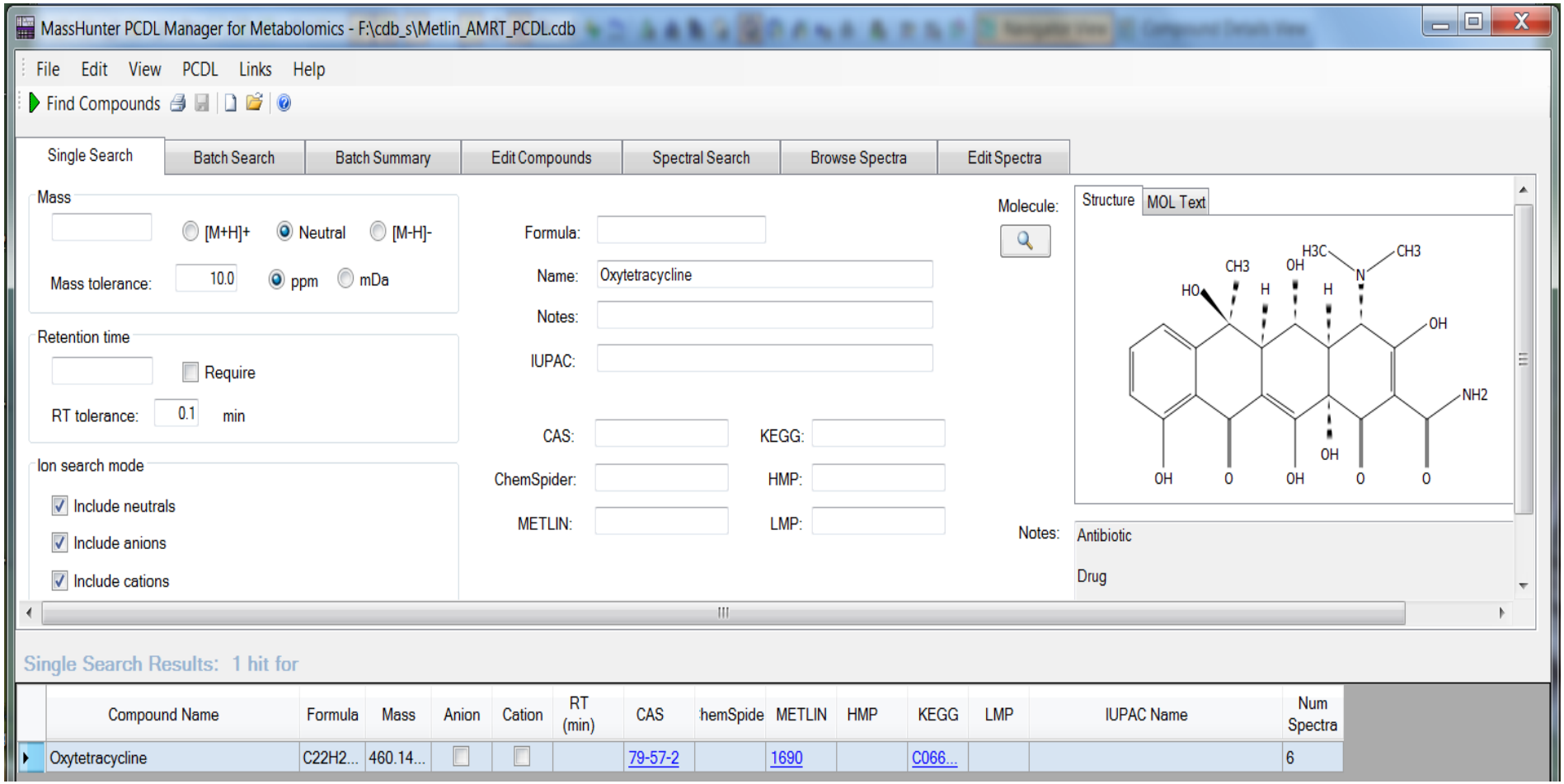


5th Step – Use MS/MS spectra and MassHunter Molecular Structure Correlation (MSC) tool to propose structures.

MS/MS structure assignment from MassHunter MSC (Molecular Structure Correlation) and Chemspider:



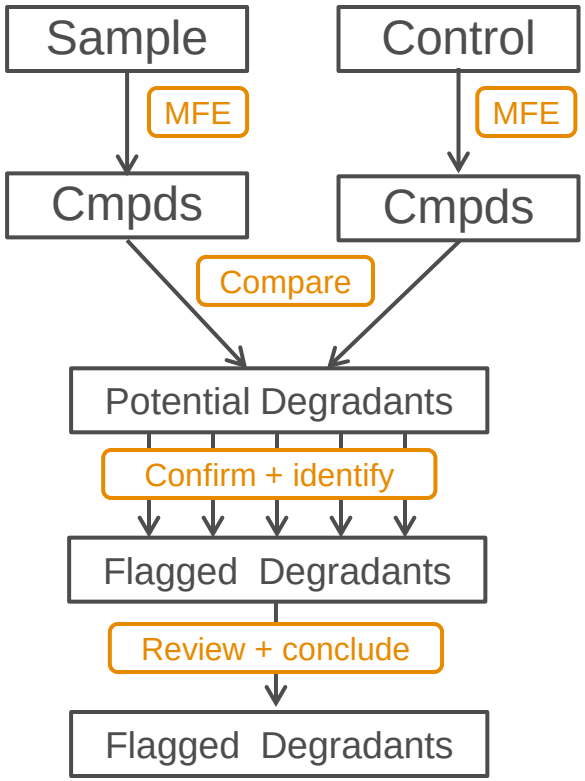
Structures can also be assigned to MS/MS spectra from User directed databases (compendium of “Learned” proprietary structures). PCDL database in MassHunter:



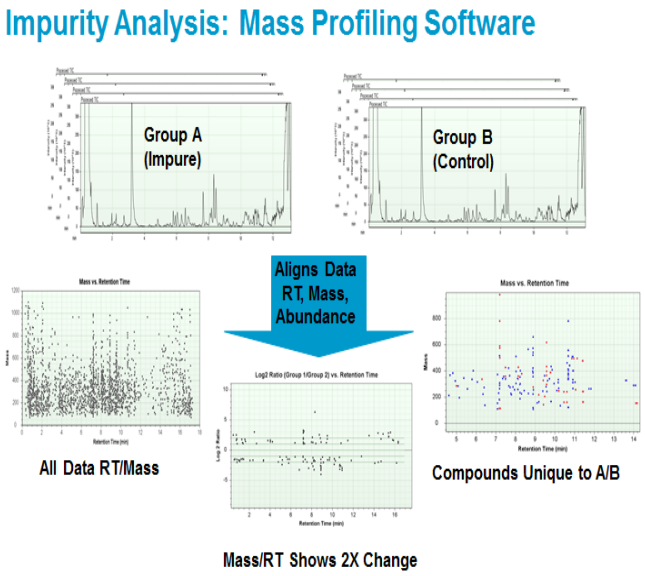
6th Step – Use MassHunter Mass Profiler (MP) tool to compare any two LC/MS files.

Untargeted approach based on MFE comparison

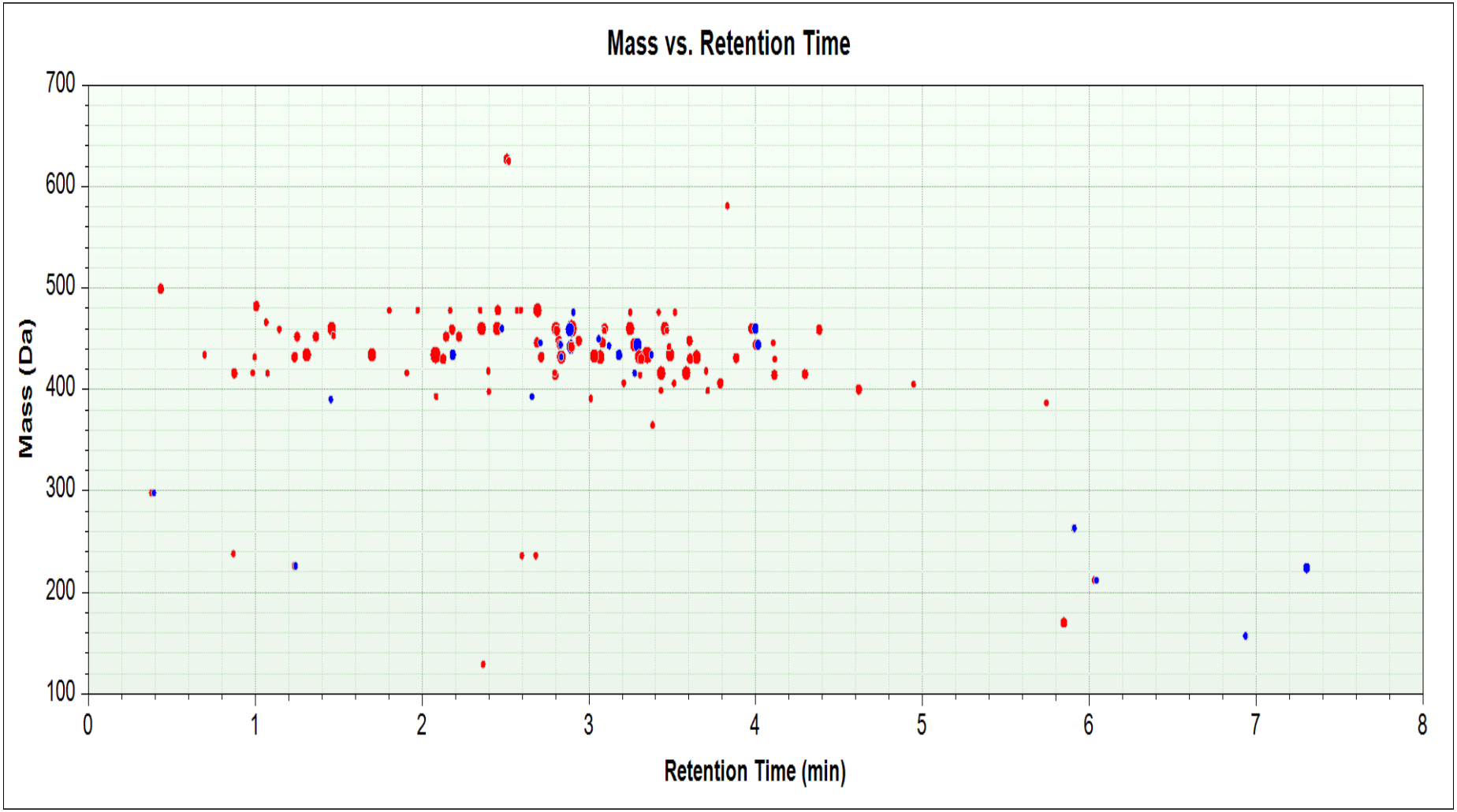
Best approach for expected and unexpected metabolites, degradants, etc



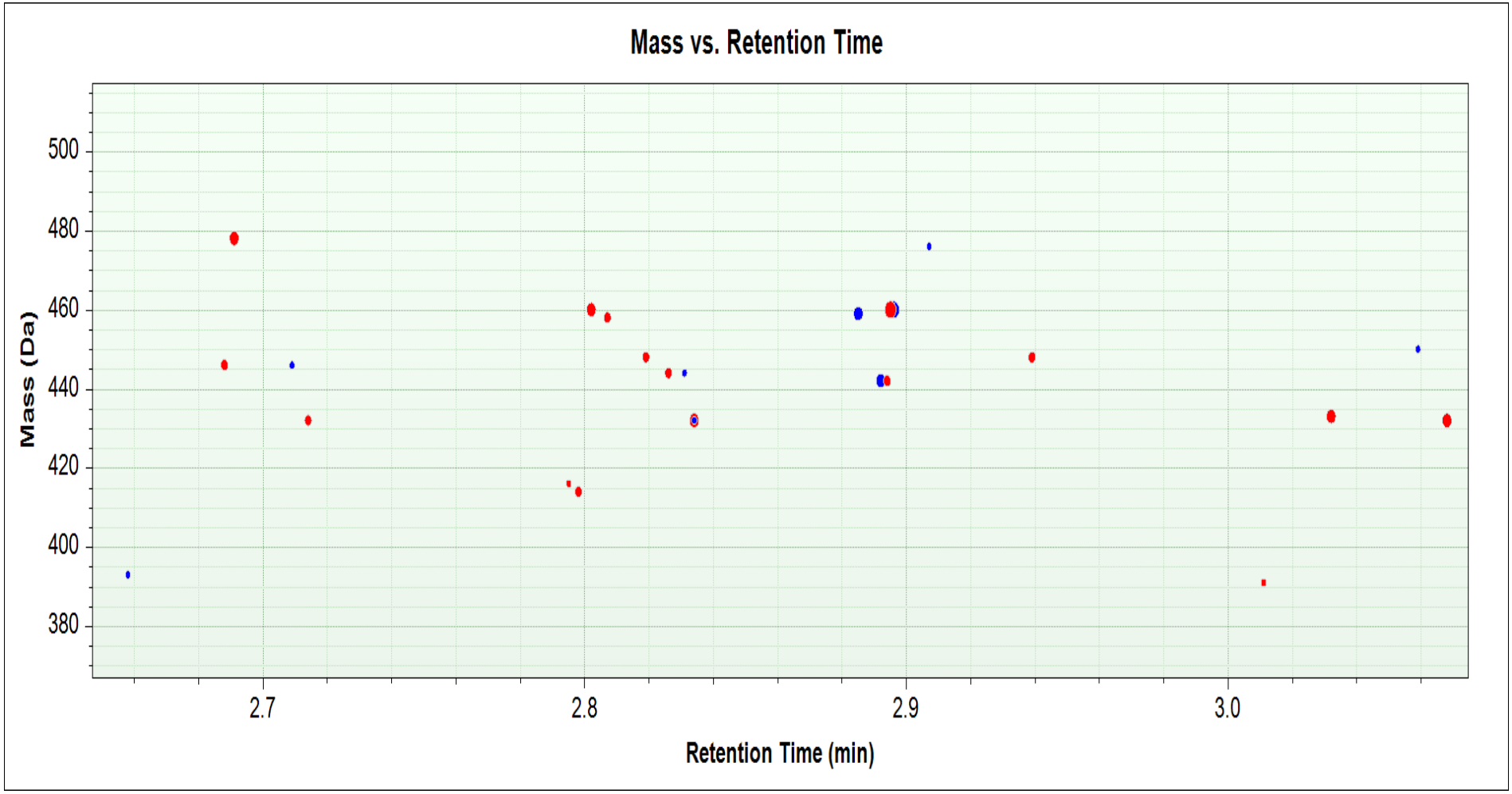
- Untargeted approach via comparison of MFE Compound lists
  - Sophisticated Sample-Control Comparison Algorithm based on Molecular Feature Extraction (MFE) allows more comprehensive detection of differences via exploitation of mass and RT resolution.



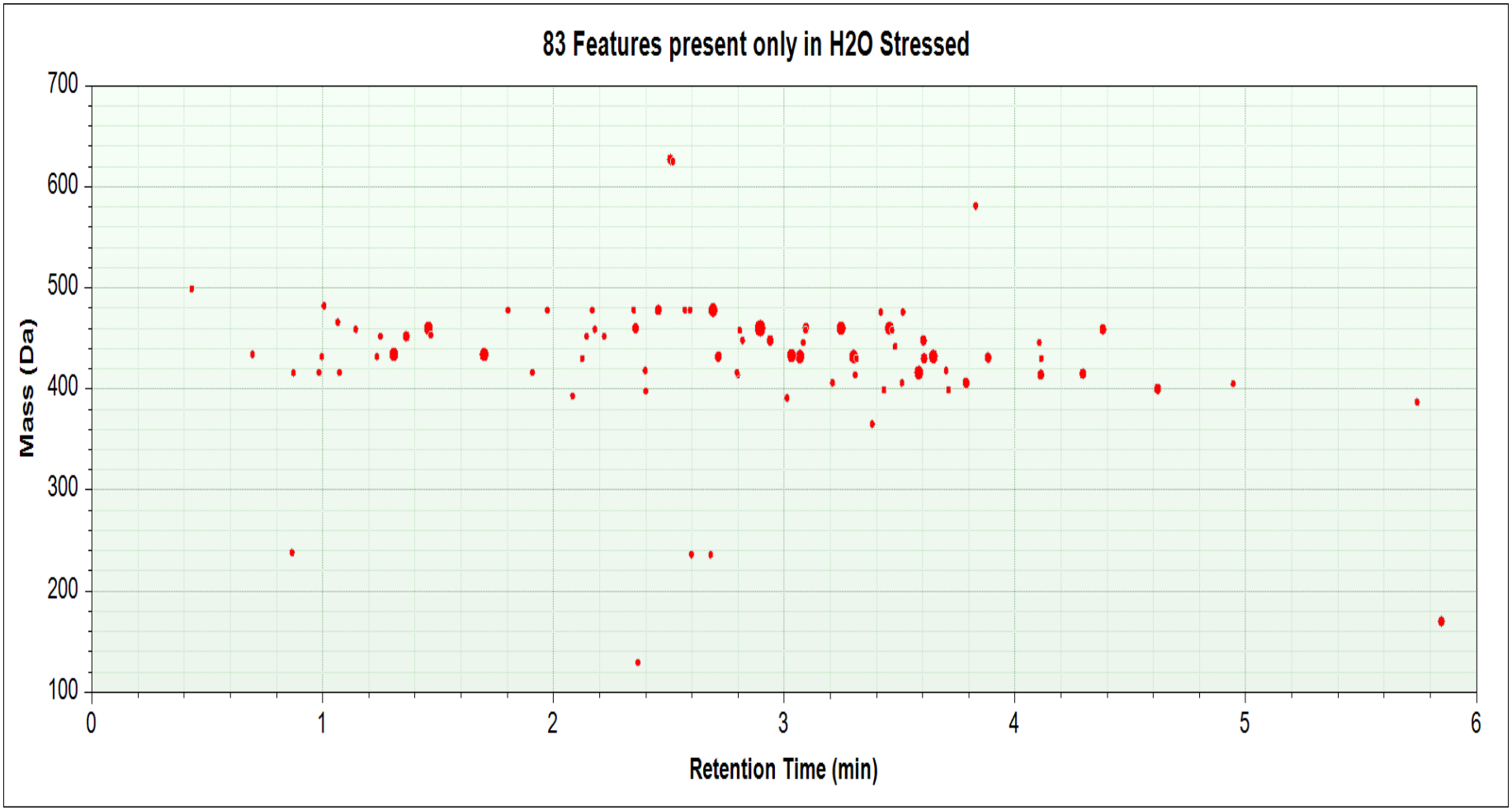
Mass Profiler allows comparison of Std. (Blue) vs H2O stressed (Red) Oxytetracycline:



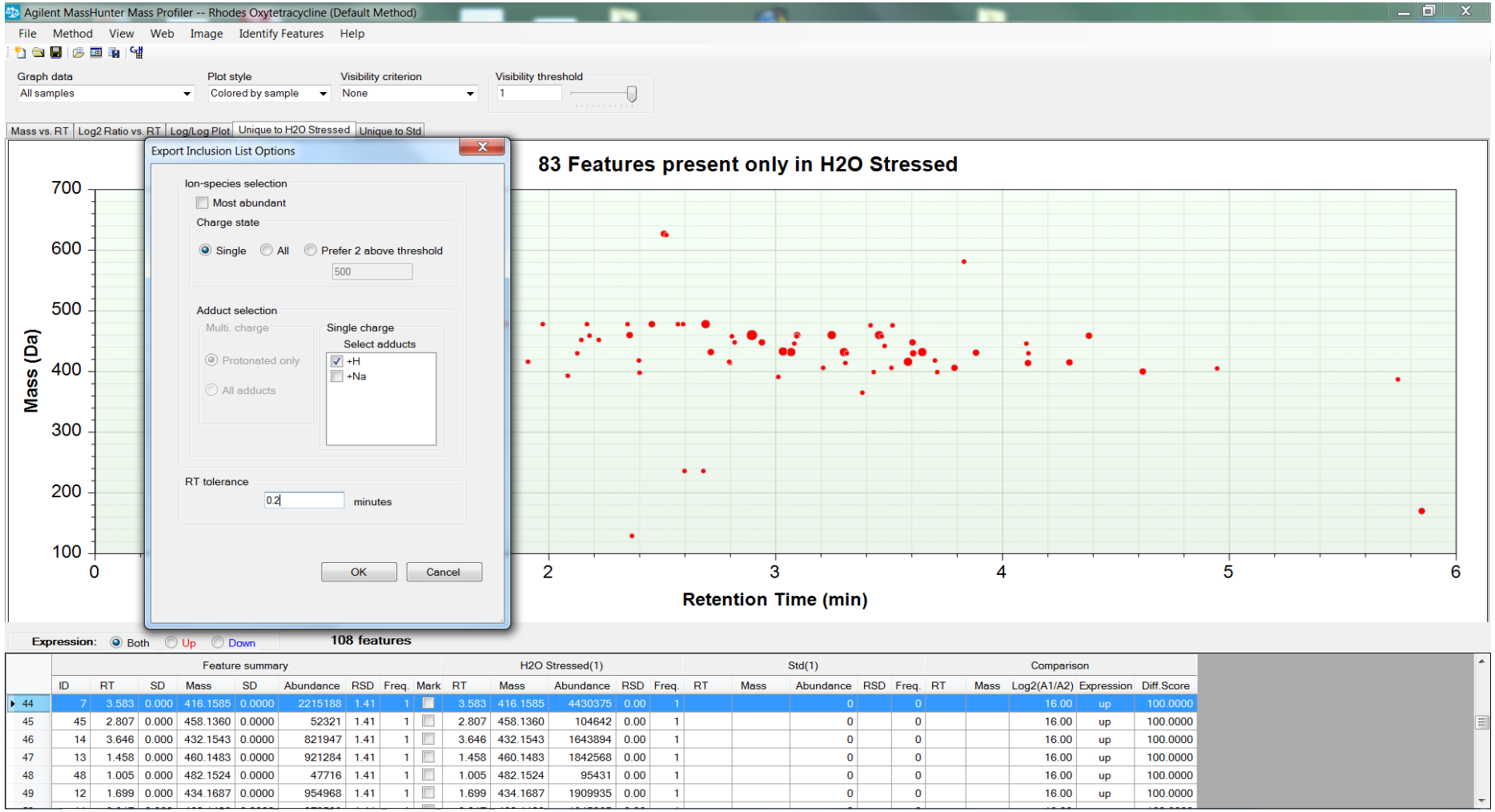
Overlaps are components common to both samples:



Mass Profiler can find components only found in H2O stressed sample (Red, degradants from API):

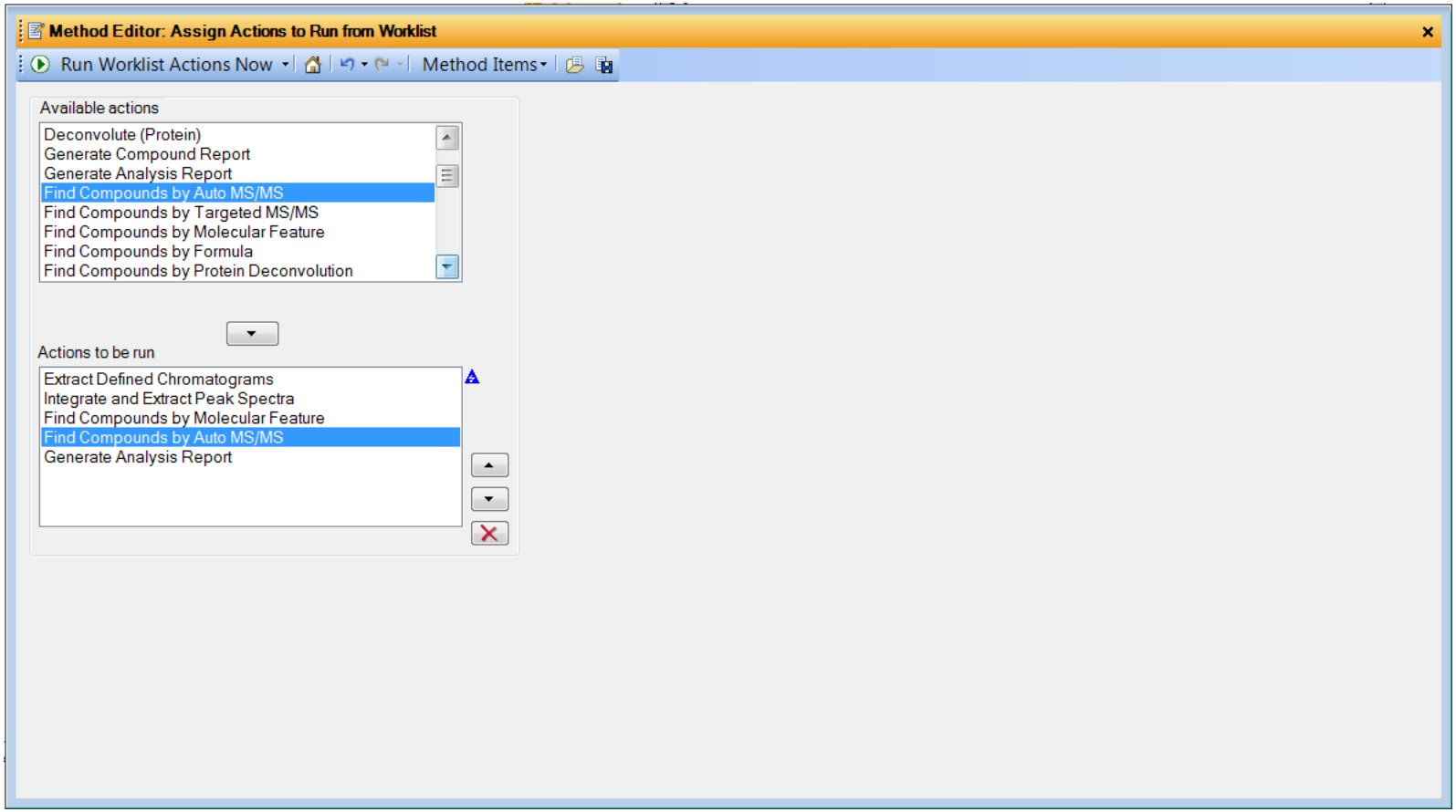


Mass Profiler can schedule further MS/MS studies if needed:



	Feature summary									H2O Stressed(1)					Std(1)					Comparison				
	ID	RT	SD	Mass	SD	Abundance	RSD	Freq.	Mark	RT	Mass	Abundance	RSD	Freq.	RT	Mass	Abundance	RSD	Freq.	RT	Mass	Log2(A1/A2)	Expression	Diff.Score
▸ 44	7	3.583	0.000	416.1585	0.0000	2215188	1.41	1		3.583	416.1585	4430375	0.00	1			0		0			16.00	up	100.0000
45	45	2.807	0.000	458.1360	0.0000	52321	1.41	1		2.807	458.1360	104642	0.00	1			0		0			16.00	up	100.0000
46	14	3.646	0.000	432.1543	0.0000	821947	1.41	1		3.646	432.1543	1643894	0.00	1			0		0			16.00	up	100.0000
47	13	1.458	0.000	460.1483	0.0000	921284	1.41	1		1.458	460.1483	1842568	0.00	1			0		0			16.00	up	100.0000
48	48	1.005	0.000	482.1524	0.0000	47716	1.41	1		1.005	482.1524	95431	0.00	1			0		0			16.00	up	100.0000
49	12	1.699	0.000	434.1687	0.0000	954968	1.41	1		1.699	434.1687	1909935	0.00	1			0		0			16.00	up	100.0000

Entire Workflow is Automated with Simple Instructions



Summary:

- An example API was found to have 59 trace level impurities, of which 17 were related to the API structure.
- Degradation of the API produced approx 60 impurities related to the API.
- MassHunter software was utilized to find automatically the impurities (MFE), calculate elemental formulas (MFG), and possible structures (MSC).
- Using MFE, impurities can be found that are not resolved by the LC separation.
- Mass Profiler software allowed for easy sample comparisons.
- The individual software steps can be linked together in a simple MH work list to automate the complete workflow.

Additional Background Information:

Agilent Products for Pharmaceutical Research:

7200 GC-Q-TOF

Bravo/
Encore

MassHunter
Software

Hi-DEF Q-TOF
6500 series

7000 GC-QQQ,
5977 GC-SQ

CE/MS

Infiniti
1290 UHPLC

SQ 6100
Series

QQQ
6400 Series

TOF
6200 series

Columns & Consumables

1200 Infinity Series HPLC, UHPLC, SFC for MS:

More workflow-automation and application-based solutions

Analytical
SFC

Bio-inert
HPLC

Nano LC
& Cap LC

Preparative
LC & LC/MS

GPC
SEC

Isocratic
LC

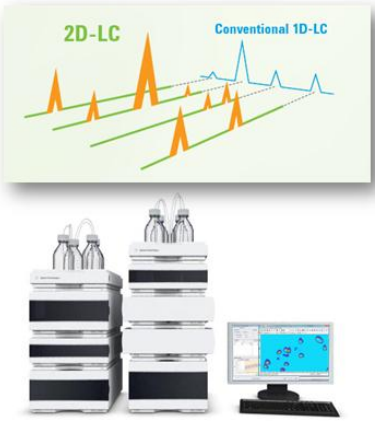
Multi-method and
Method Development

High-throughput
LC & LC/MS

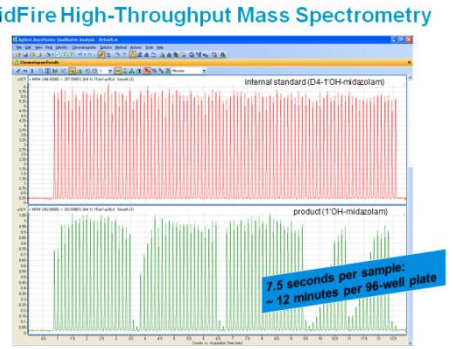
Nanoflow
HPLC-Chip MS

Sample Introduction Choices for your Agilent LC/MS – *More than just HPLC/UHPLC*:

New multi-dim LC choices with valve automation:

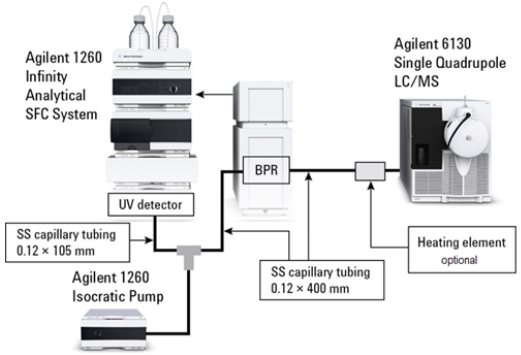


HT SPE-MS Agilent RapidFire



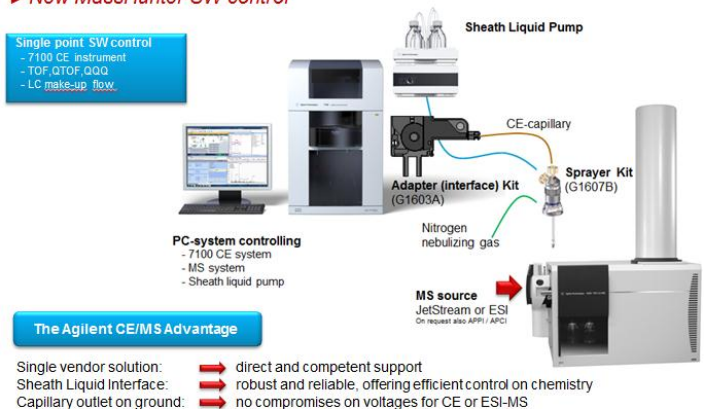
GC-APCI MS

SFC/MS: From SQ to QTOF



Agilent CE/MS Setup

► *New MassHunter SW control*



LC/MS Ion Sources – Many Choices

- Widest choice of sources from any manufacturer
  - Electrospray (ESI)
  - Agilent JetStream ESI
  - Nanoelectrospray (nanoESI)
  - APCI
  - APPI
  - Dual ESI sources for TOF and Q-TOF
- Multimode Source
  - ESI and APCI combined in one source
  - Simultaneous operation
- HPLC-Chip/MS
  - Nanoelectrospray made easy
  - Reproducible results
  - Sample processing on chip
- Most sources are interchangeable between MS platforms

Ionize Almost Anything!