Agilent TOF Screening & Impurity Profiling

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LC/MS Small Molecule Workshop

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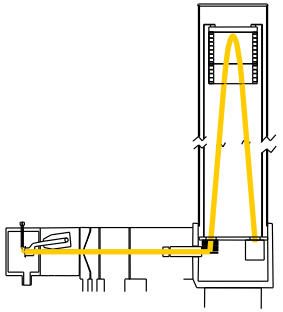
Review: Technology for Accurate Mass Analysis: TOF – LC/MS

•Mass measurements accurate to several decimal places (rather than "nominal" or "unit" mass measurements of ± 0.1 Da), due to *high resolution* mass analyzer.



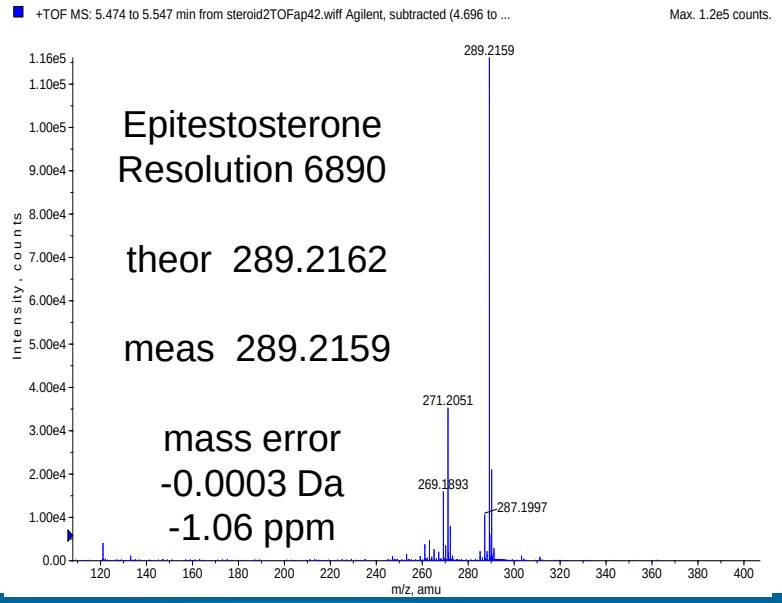
•Most Common Uses:
- Rapid selective and specific screening for target compounds
- Confirmation of molecular formula for proposed compounds
- Calculation of possible molecular formulae for unknowns

Definition of Mass accuracy (mass error)

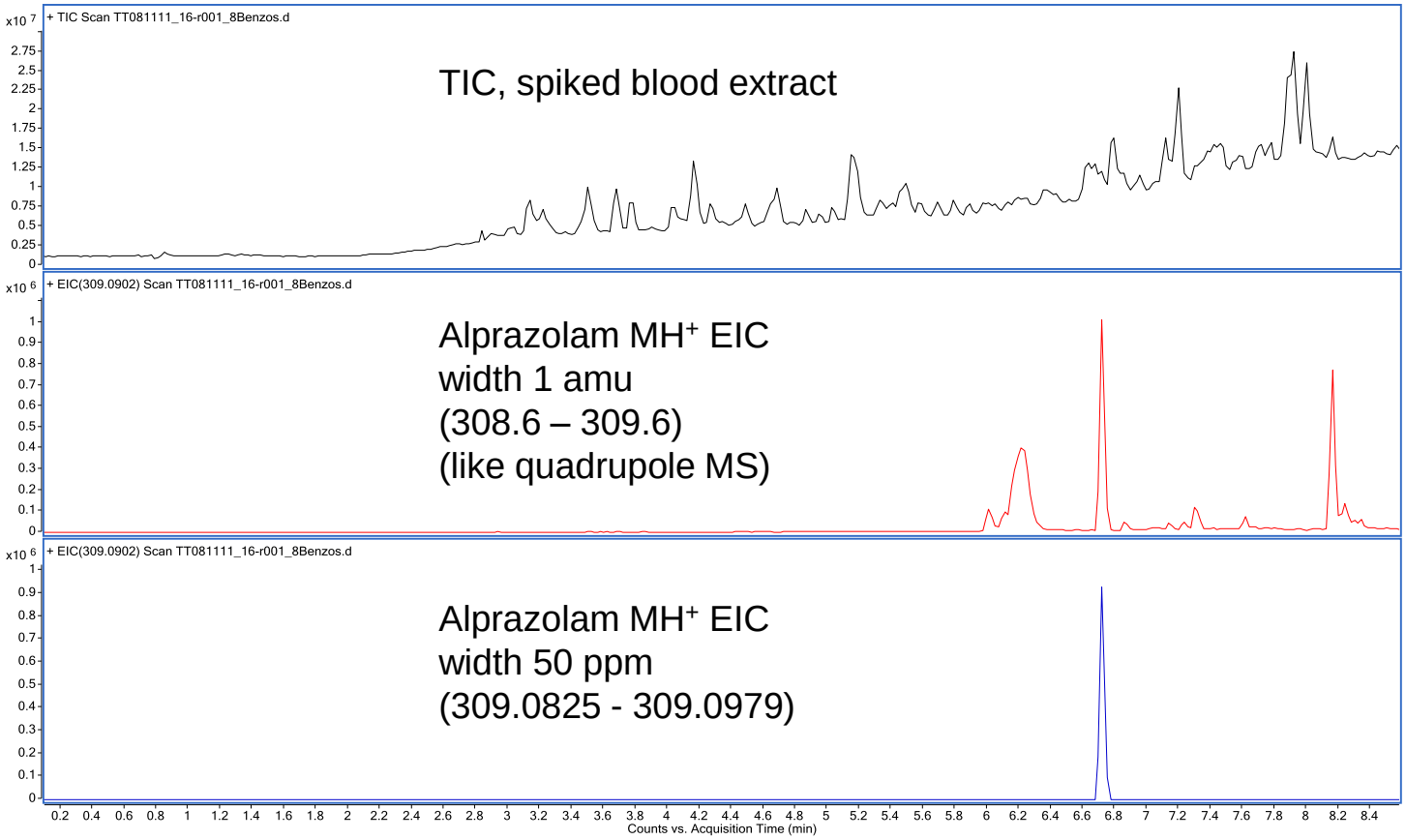
$$\text{Mass accuracy} = \left(\frac{\text{measured - theory}}{\text{theory}} \right) \times 10^6$$

(1000.001-1000.000)/1000) x10^6 = 1 ppm

Therefore: 0.001 Da (1 mDa) error
@ MW 300 Da is approximately 3 ppm



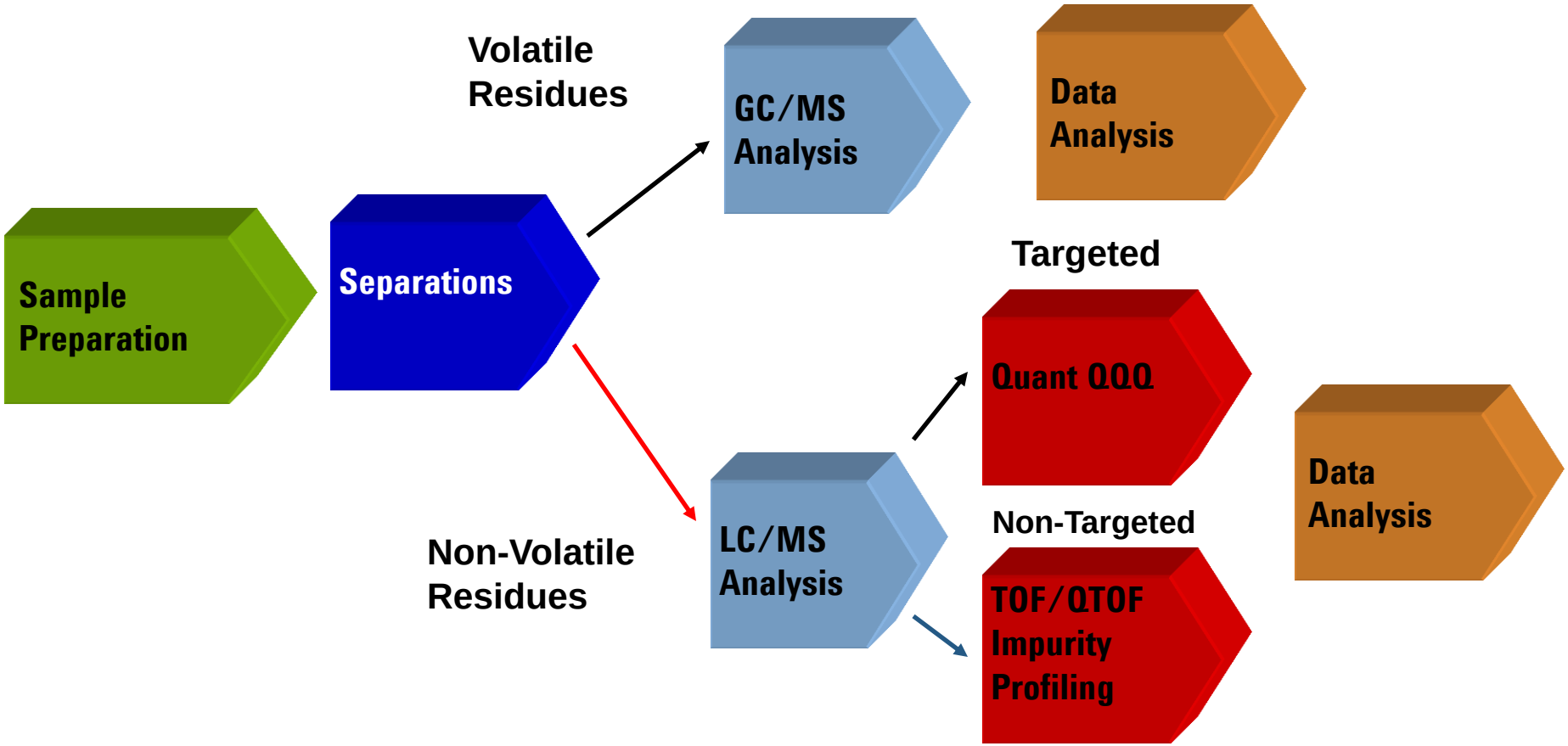
Specificity of narrow-mass EICs: locating compounds



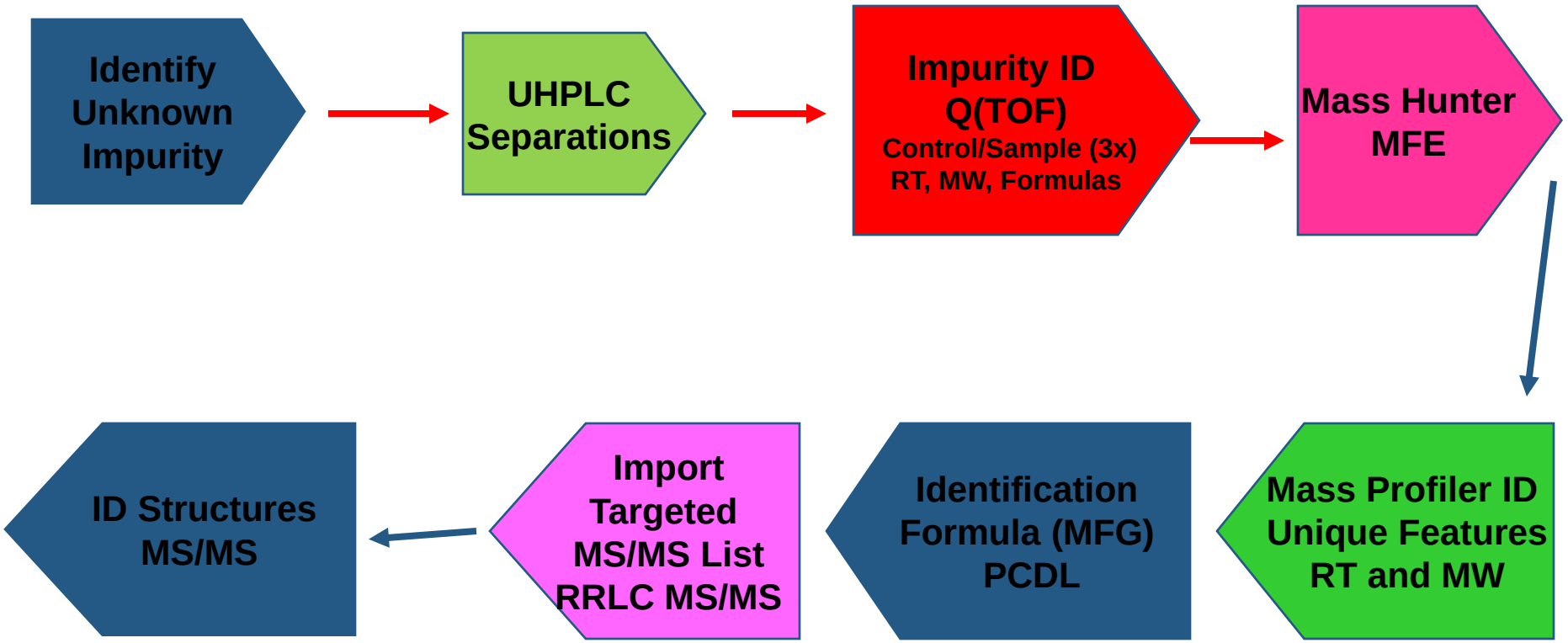
TOF's unique advantage over MS/MS: all-scan data allows retrospectively finding compounds not originally sought

Agilent's Solution for Impurity Profiling

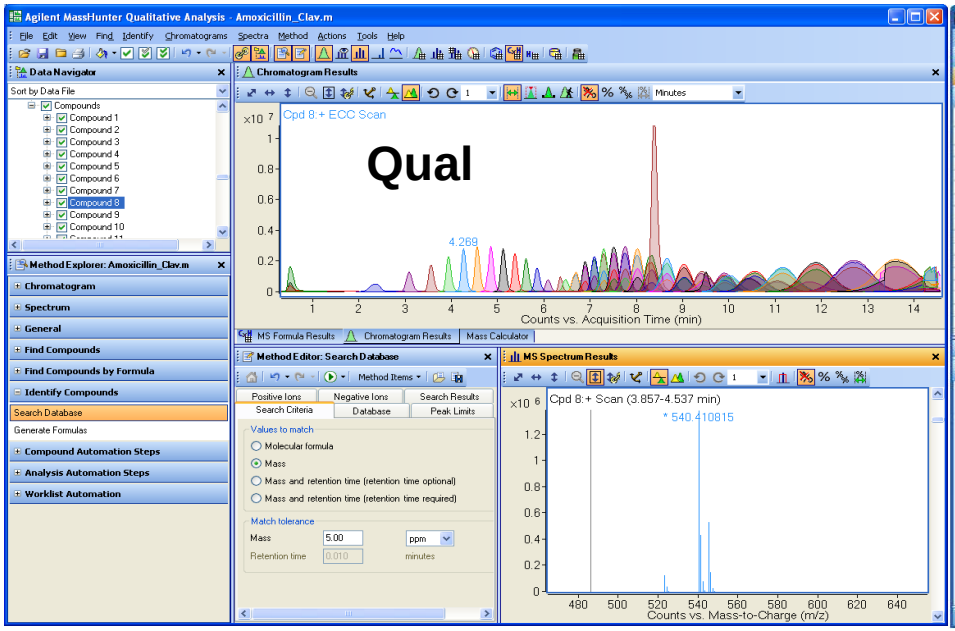
Objective: Detect low-level Organic Impurities in Drugs: Tablets, Capsules, Suspension, Transdermal, Meter Does Inhalers (MDI)

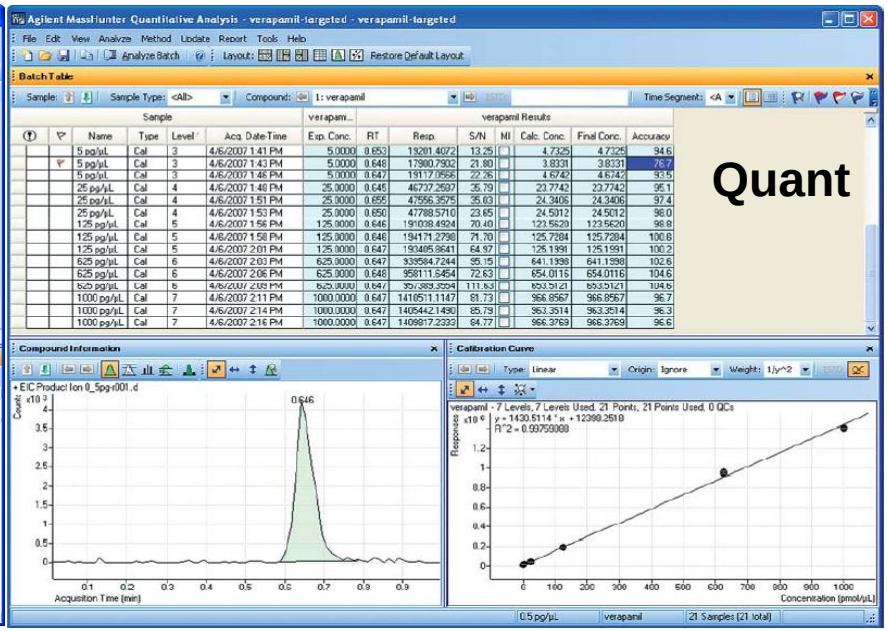


The LCMS Impurity Profiling Unknown Workflow

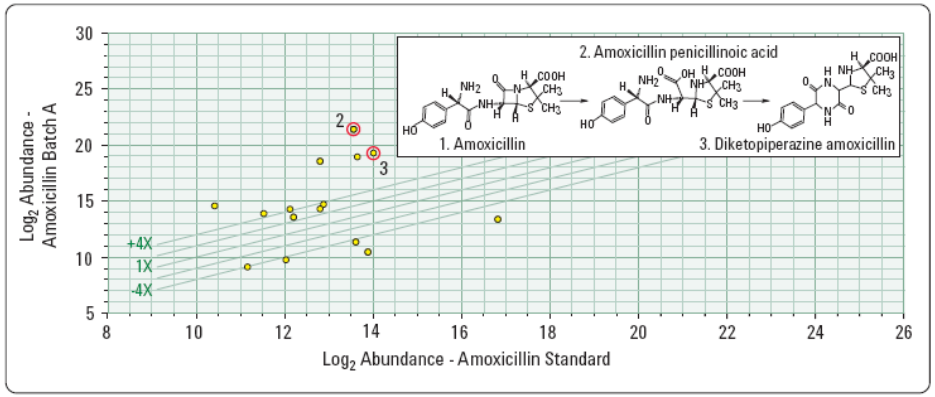


Agilent Suite of MassHunter Products



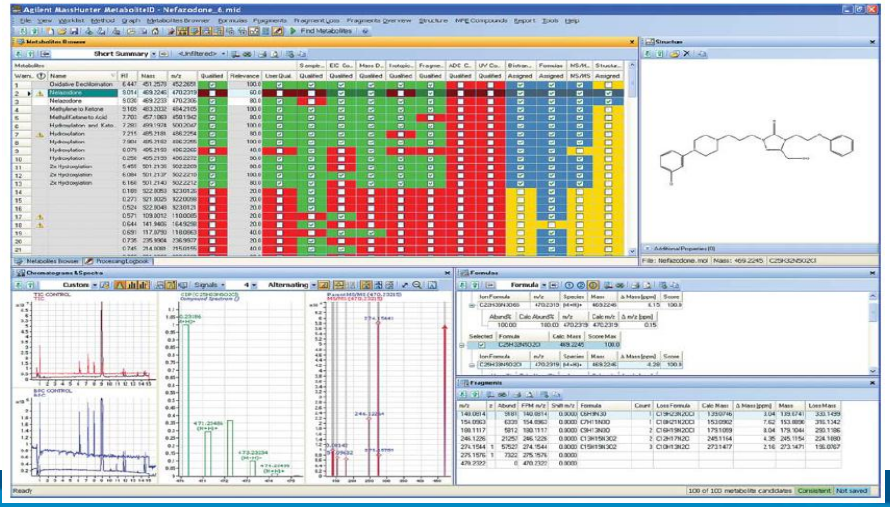


Mass Profiler



Comparison of amoxicillin and an amoxicillin standard using MassHunter Profiling software reveals two impurities (degradants) tentatively identified as amoxicillin penicillinoic acid (2) and diketopiperazine amoxicillin (3).

MetID



What, How Many, How Much, In What

Information Needed to Optimize Analyses

- **What** are you trying to detect
  - Compound Class, Polarity, Solubility, Ionization Methods
- **How Many** Compounds
  - LC Separations (C18, ANP), Mobile Phases-Buffers, Ion Suppression, Matching LC and MS Peak Widths
- **How Much** and Over What Range
  - Trace level detection (LOD/LOQ) vs. Impurities
  - Wide Dynamic Range – Extended Dynamic Range Model
  - Co-eluting low molecular weight analytes – High Resolution Mode
- **In What** Matrix
  - Complexity of Matrix, Major Components, Ion Suppression, Salts
  - Co-eluting components – what instrument state to use

The Match Game….

Choosing The Mass Spectrometer for the Task

Sensitivity, Selectivity, Identification..

Unknown Identification
Accurate Mass MS, MSMS
MS/MS Sensitivity

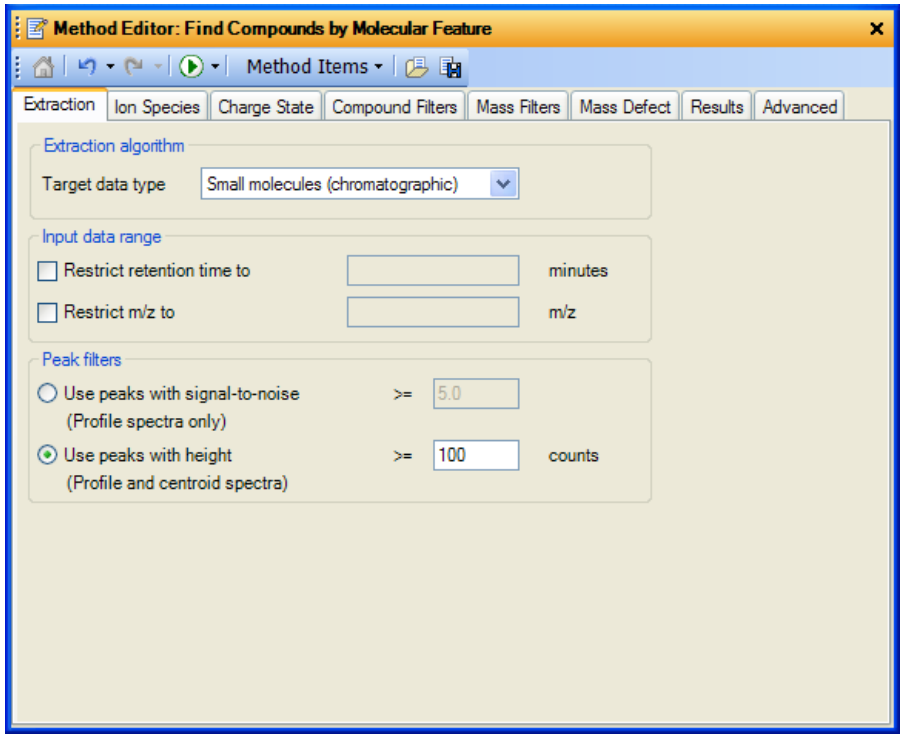
Unknown Identification
Accurate Mass MS

6490 QQQ

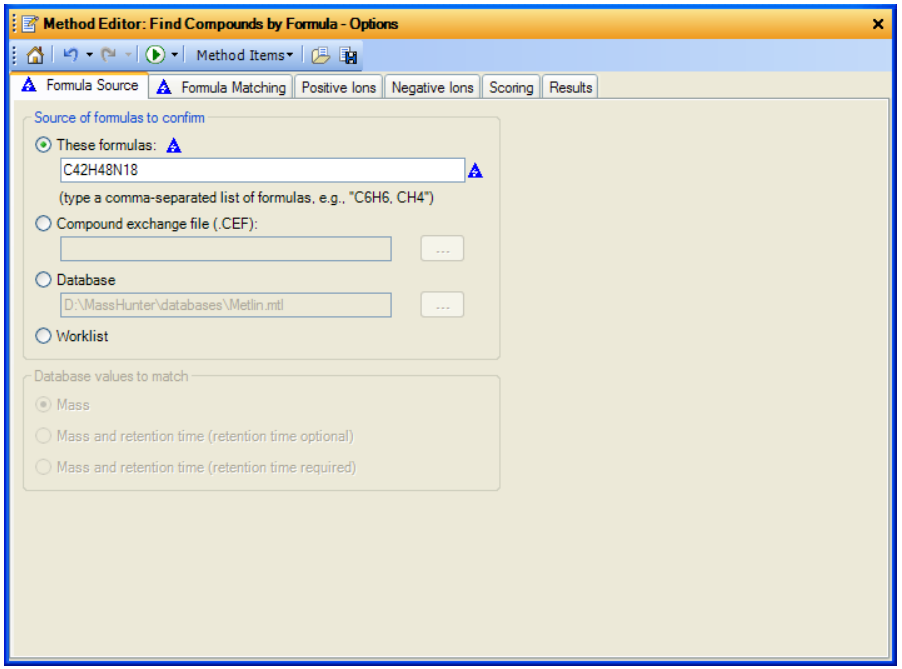
Target Analysis
Enhanced Sensitivity by MS/MS

Find Compounds Functions in MassHunter Qual

Find by Molecular Feature (MFE)



Find by Formula/ Ion
FBF & FBI



“Naïve”

“Targeted”

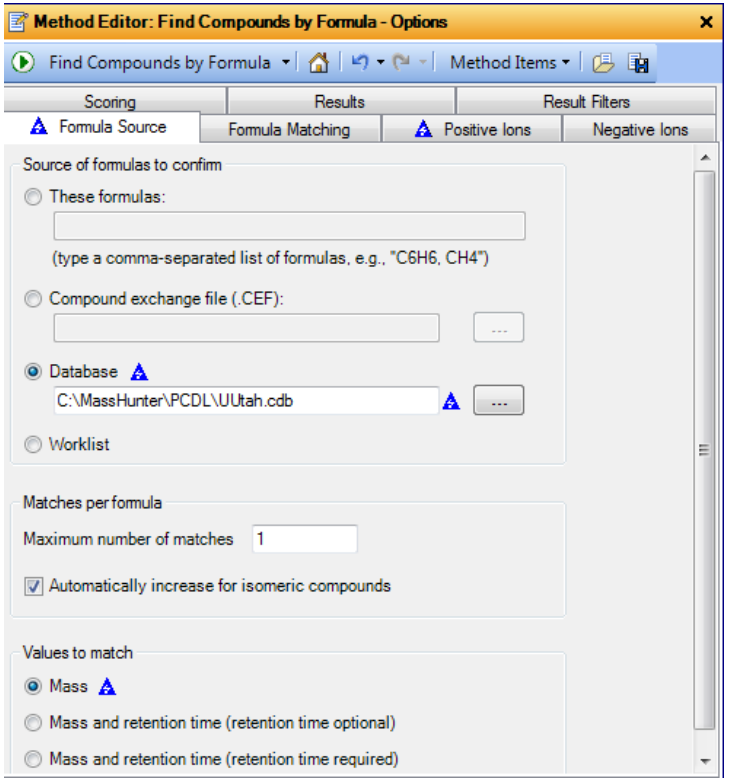
2 Data Processing Approaches using Peak Picking Algorithms

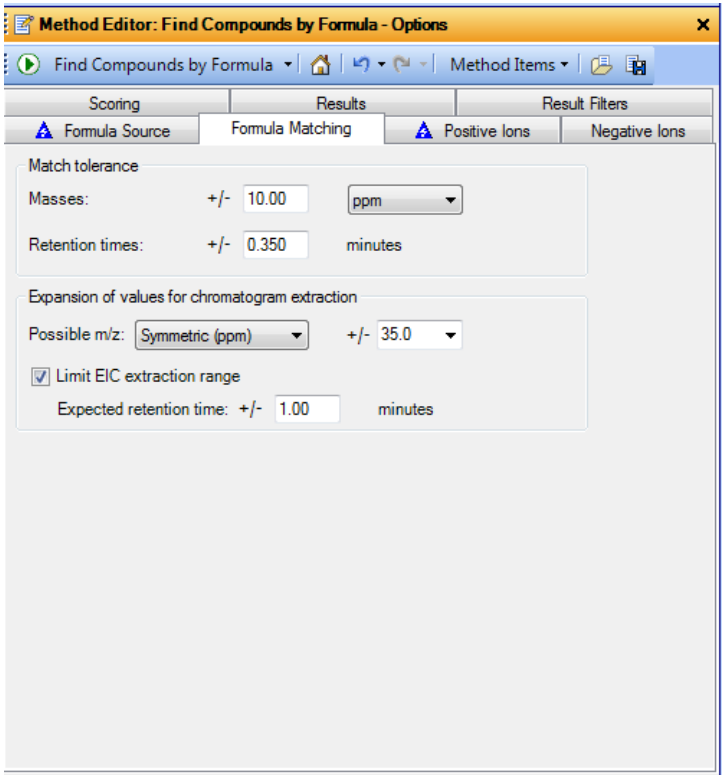
- **Find by Formula**
- **Find by Molecular Feature**

Data Processing Approaches

Approach 1:

1. Run ***Find by Formula***
2. Advantage: no threshold setting allows data to be extracted from the “grass”
3. Disadvantage: will only show results for compounds in a compound database or specified





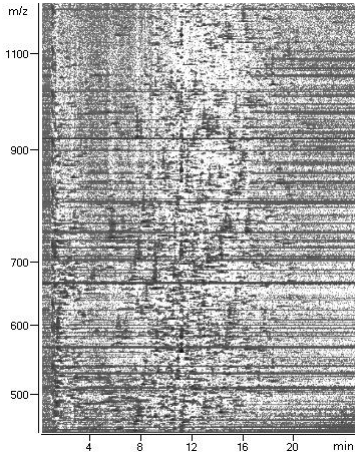
Data Processing Approaches

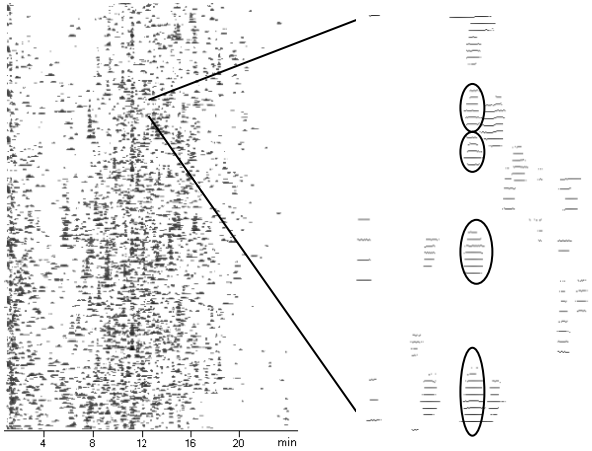
Approach 2:

1. Run Find Compounds by ***Molecular Feature Extractor***
2. Search PCD for matching compounds
3. Run Generate Formulas from Compounds for unknowns
4. Advantage: will show information for unknowns

Molecular Feature Extractor (MFE)

Transforming 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

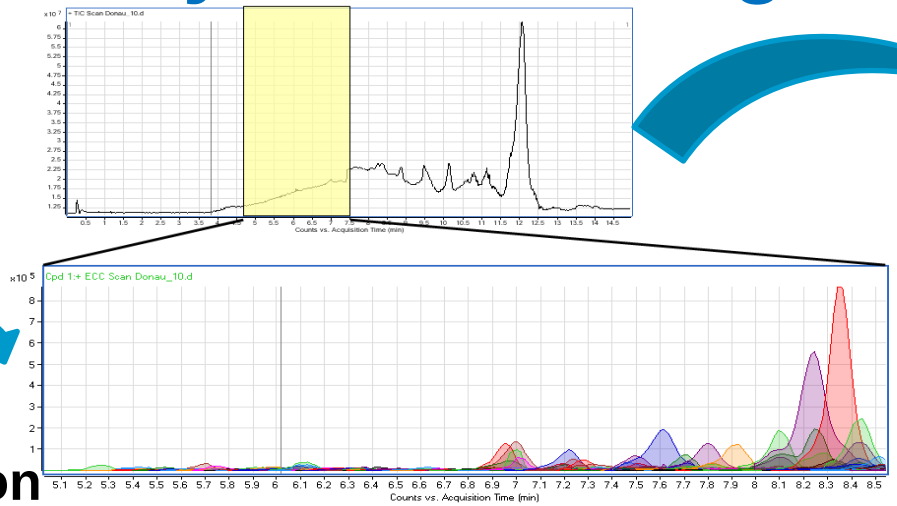
	RT	mass	abund.	height	#ions
463	7.715	1271.5948	11146	1329	3
464	7.716	1579.7000	1223	188	2
465	7.716	748.3860	18753	1216	4
466	7.718	1970.7935	2788	281	3
467	13.242	2826.4049	11853	662	4
468	13.244	2814.4034	60168	1698	8
469	13.245	2816.4119	8707	680	3
470	13.246	2331.1504	3191	291	3
471	13.247	1407.6968	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	685	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*

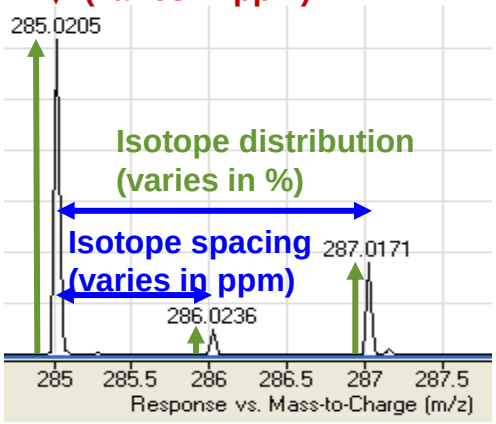
- Looks at Mass spectral data first → Groups co-eluting isotopes & converts to neutral mass
- Checks that there is a chromatographic response
- Groups all charge states, adducts, etc. associated with a given feature into a “peak volume”
- Feature consists of an accurate neutral mass, a retention time, and a peak volume

Molecular Feature Extraction (MFE): Proprietary Peak Finding Algorithm



Scoring based on

Monoisotopic mass (varies in ppm)



species	RT	m/z	mass	abund.
M	8.162		342.1467	130643
M+H	8.165	343.1547	342.1474	11889
M+H+1	8.162	344.1581		2290
M+H+2	8.157	345.1748		369
M+H4N	8.164	360.1807	342.1469	8420
M+H4N+1	8.156	361.1893		1227
M+Na	8.162	365.1359	342.1466	75678
M+Na+1	8.163	366.1394		15324
M+Na+2	8.162	367.1429		1901
2M+Na	8.164	707.2810	342.1459	4629
2M+Na+1	8.162	708.2860		1808
2M+Na+2	8.173	709.2895		336

Approach 1: Find by Formula (FbF)

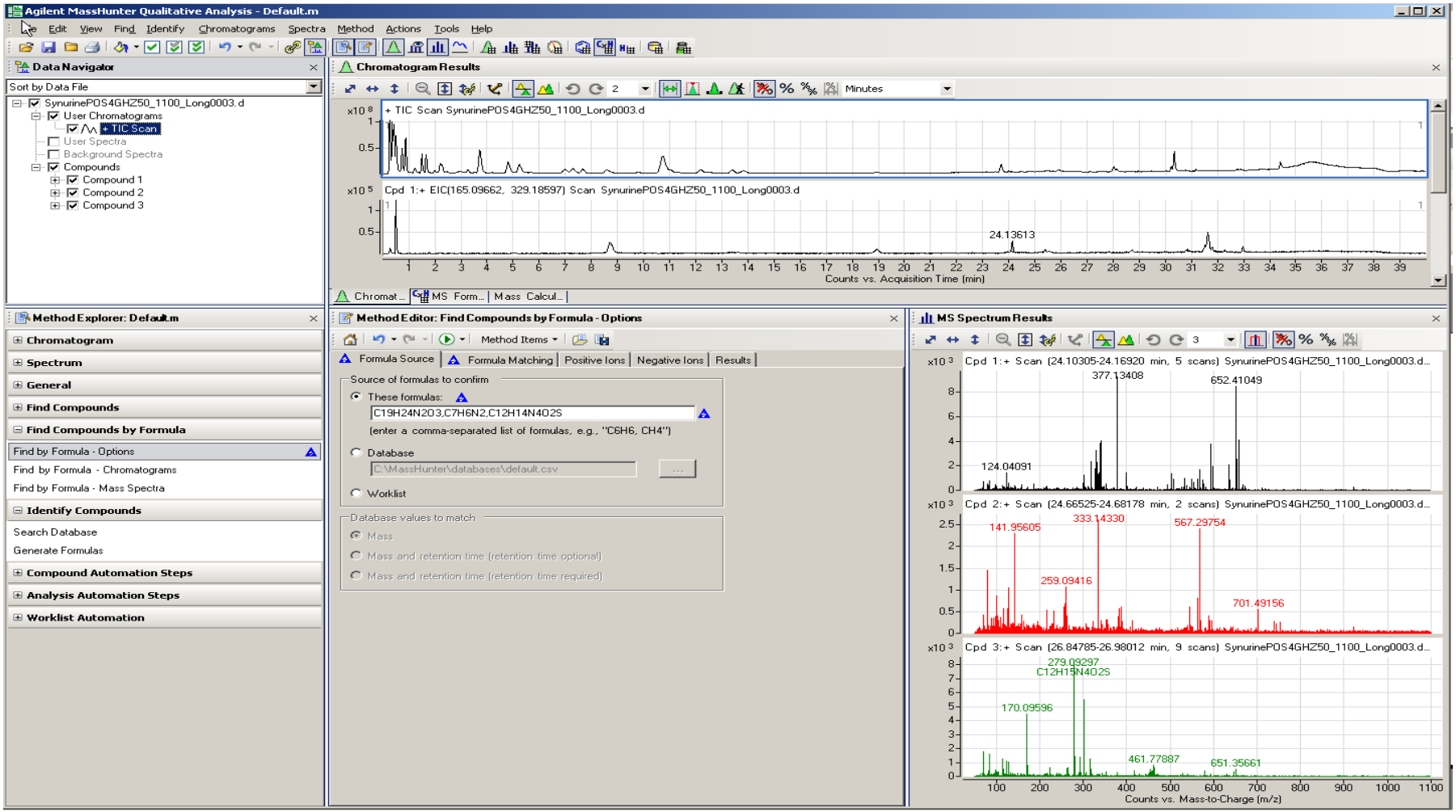
Targeted Screening Criteria for Method

Compound is "found" if:

- Retention time is within ±0.15 minutes of standard (typical match within ≤ 0.05 min)
- Mass error for adduct is within ±15 ppm of theoretical (most are < 5 ppm)
- Target compound Score is > 50/100
  - Combination of retention time match, mass matches and isotope abundance match
  - Weighting for isotope match and abundance can be adjusted to compensate for chemical interferences with low-abundance isotopes if overly affecting Score

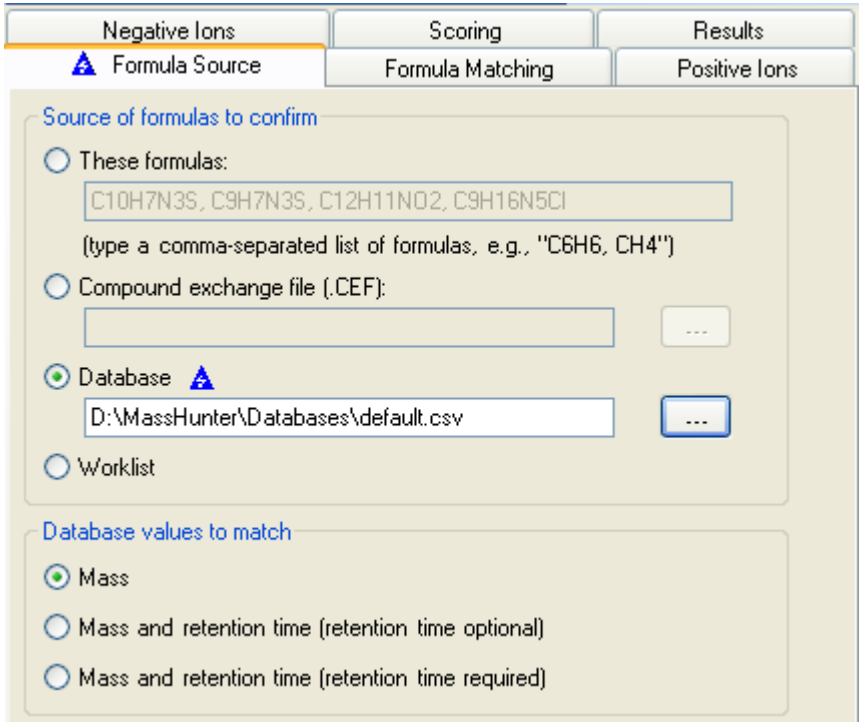
Find Compounds by Formula (FBF)

Target Compounds Adducts, Dimers, Trimers



Find Compounds by Formula Parameters

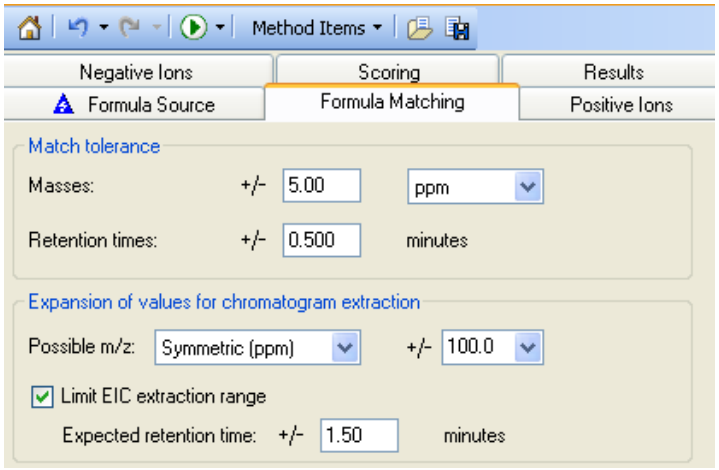
1. **Formulas Separated by Commas**
2. **CEF File Format**
3. **Databases** (Custom CSV/Metlin)
4. **Import from Worklist**
   - In Acquisition Worklist
   - Add Neutral Formulas Imported

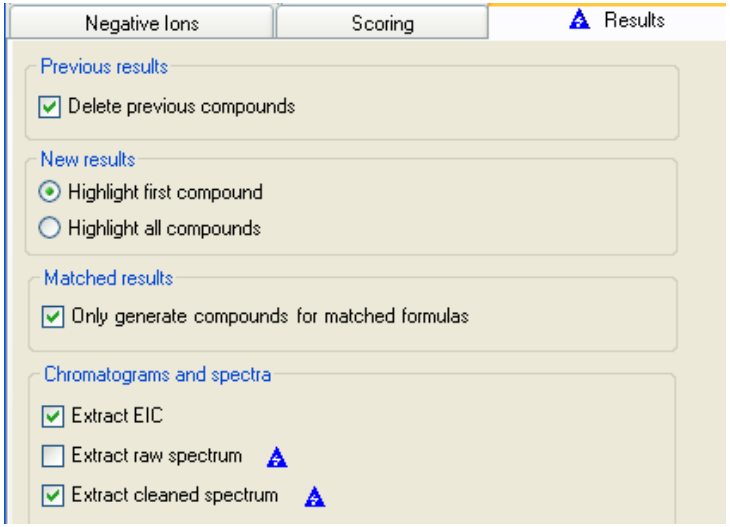


Method	Azoxystrobin	Carbaryl	Linuron	propazine	Tetraconazole	Thiabendazole	Triclclazole
Default.m	C22H17N3O5	C12H11NO2	C9H10Cl2N2O2	C9H16ClN5	C13H11Cl2F4N3O	C10H7N3S	C9H7N3S

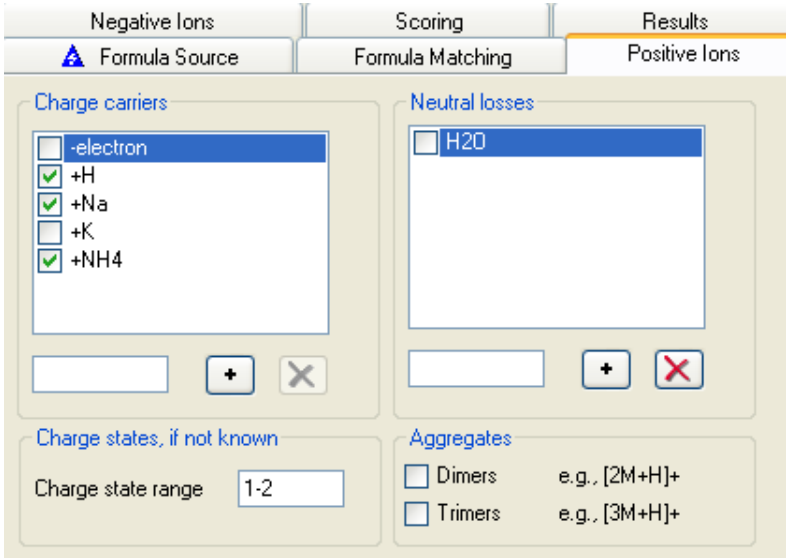
Find Compounds by Formula Parameters

Mass and RT Matching Criteria



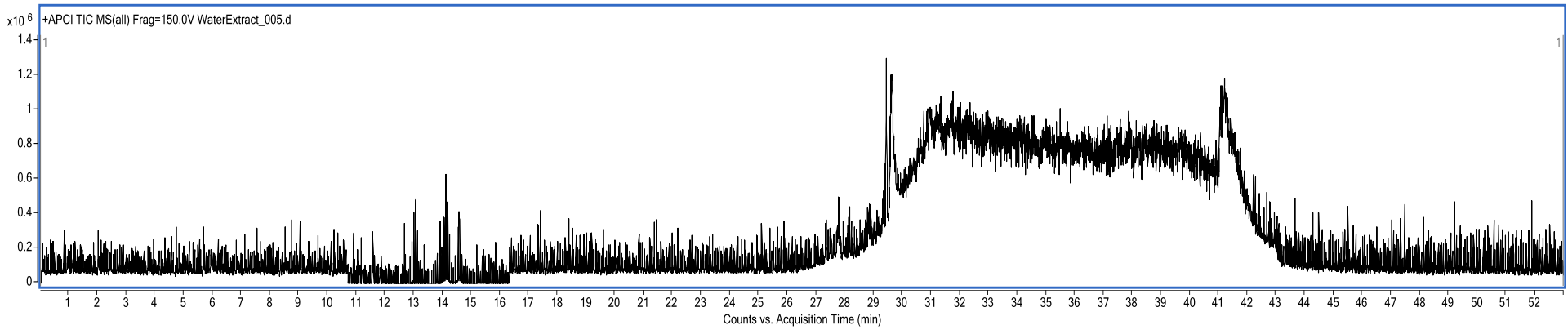


Pre-Specify Adducts

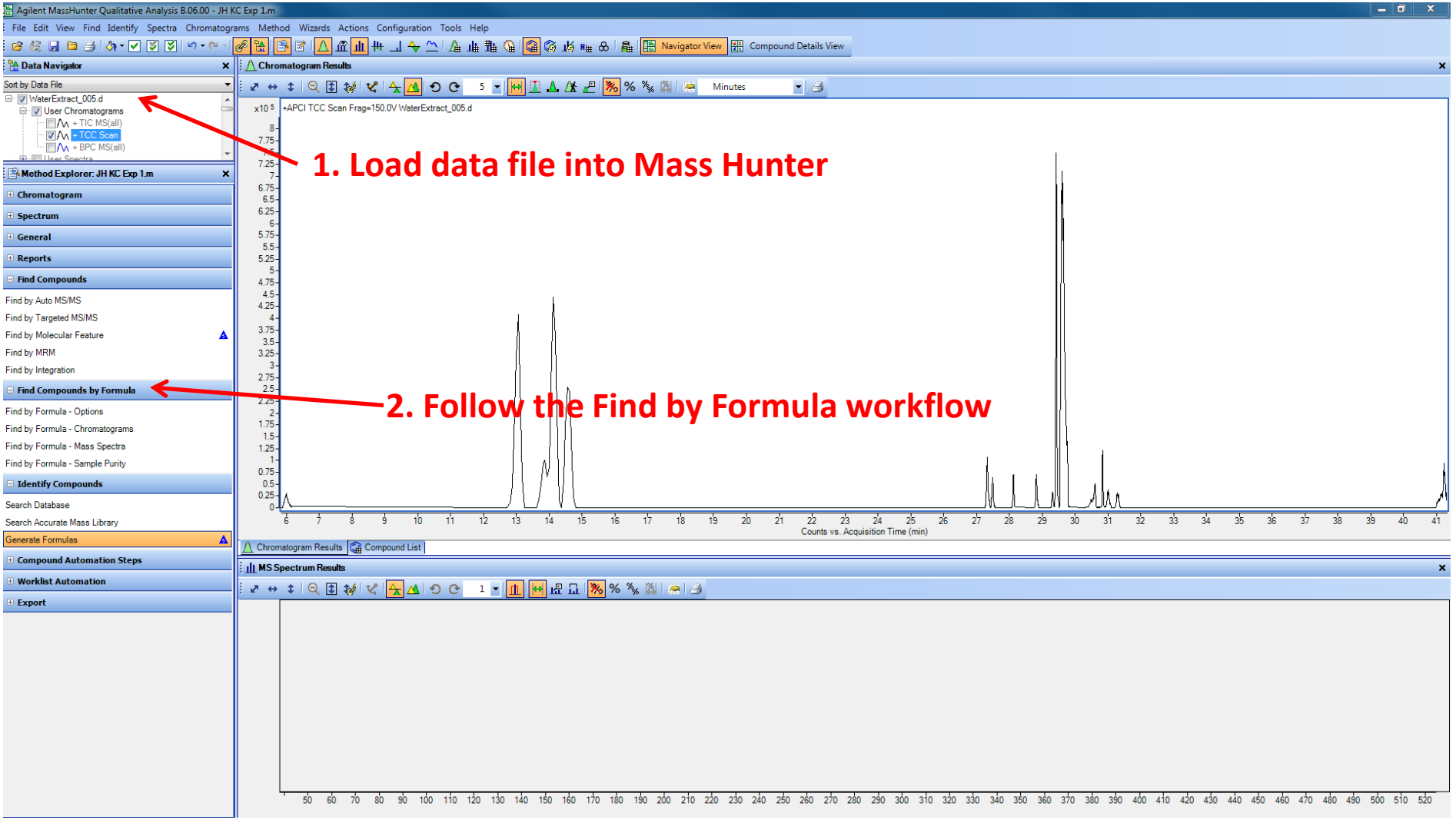


Check “Electron” to find Quaternary Amines

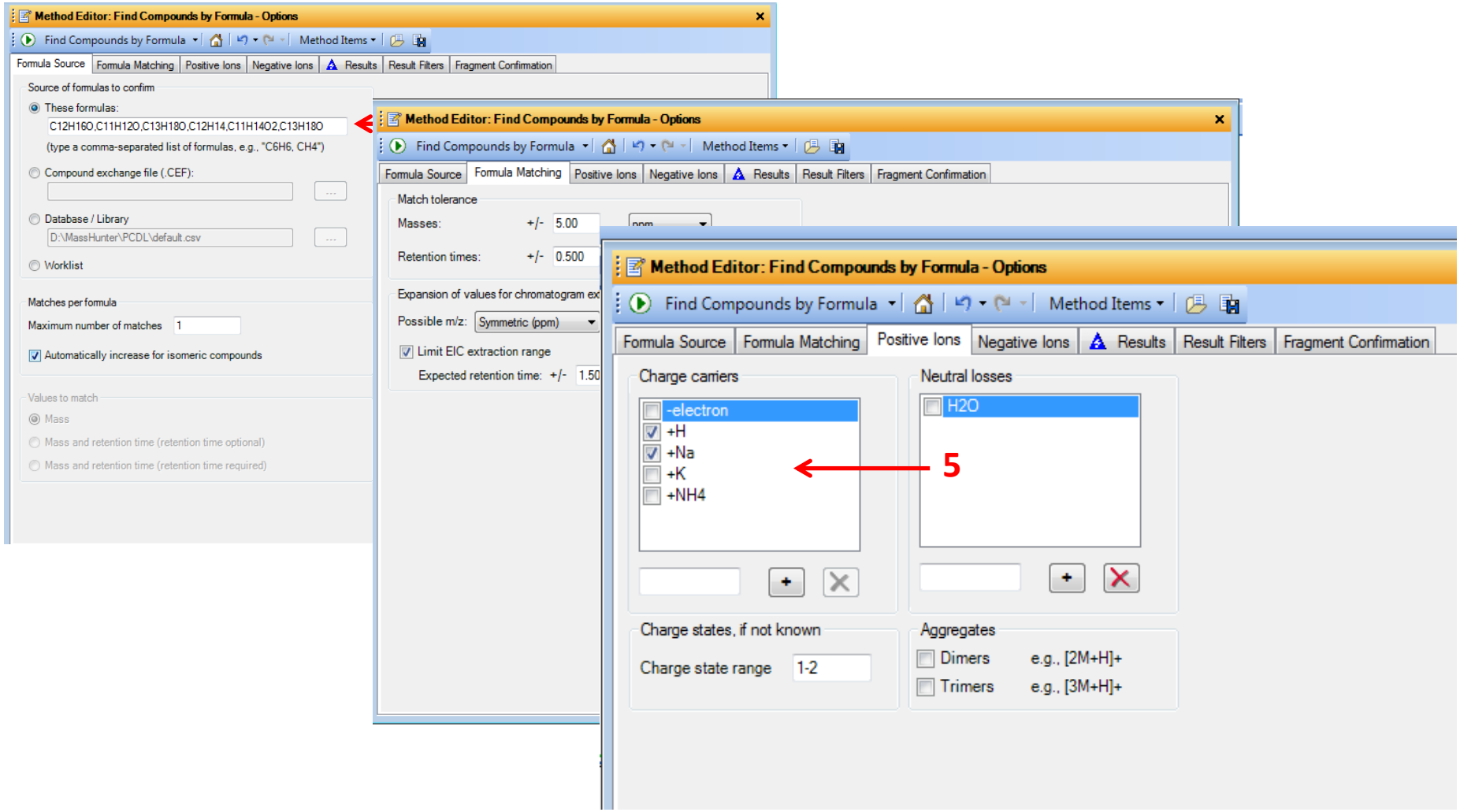
Qualitative Analysis Water Extracts: TIC +



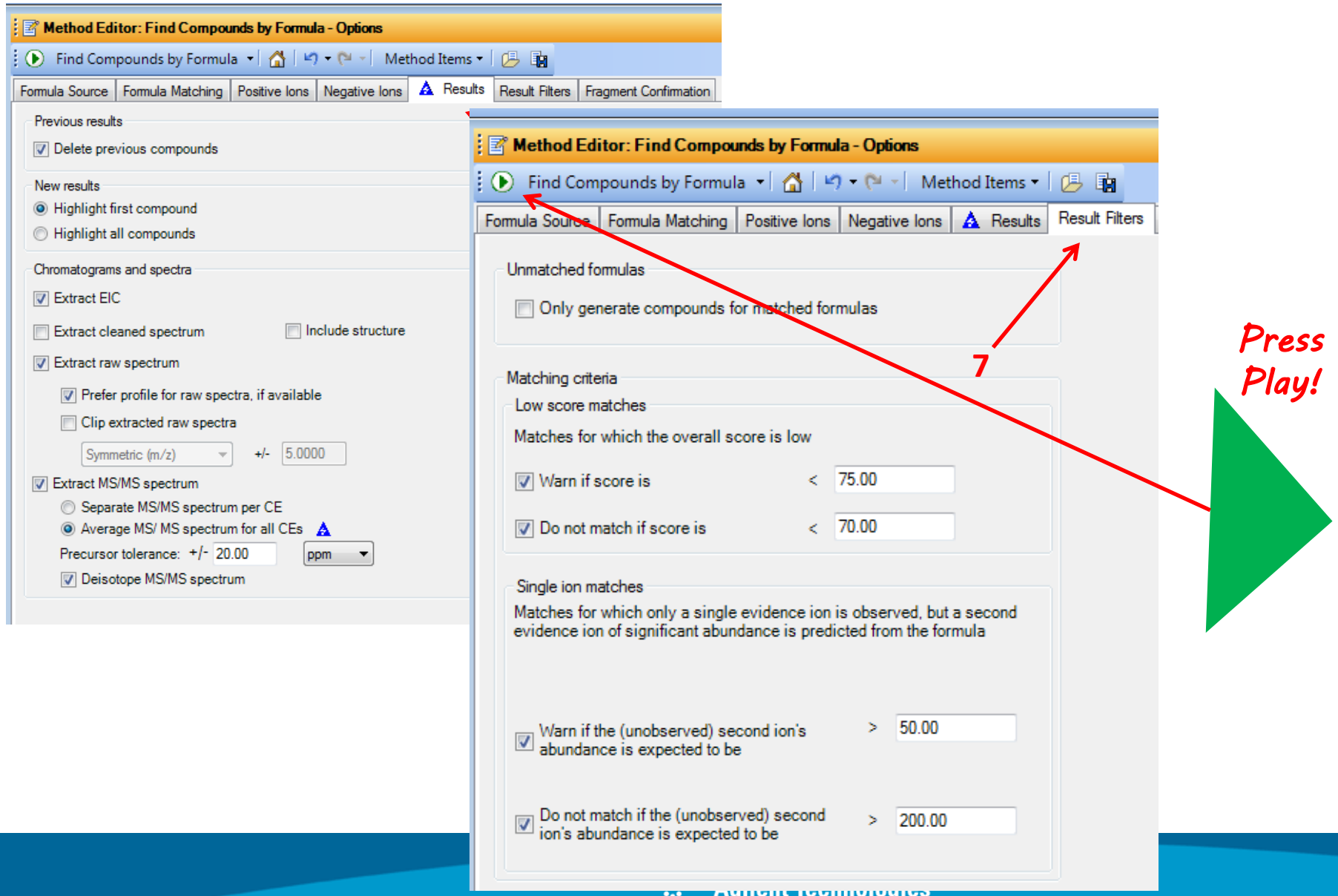
Qualitative Analysis Water Extracts: FbF



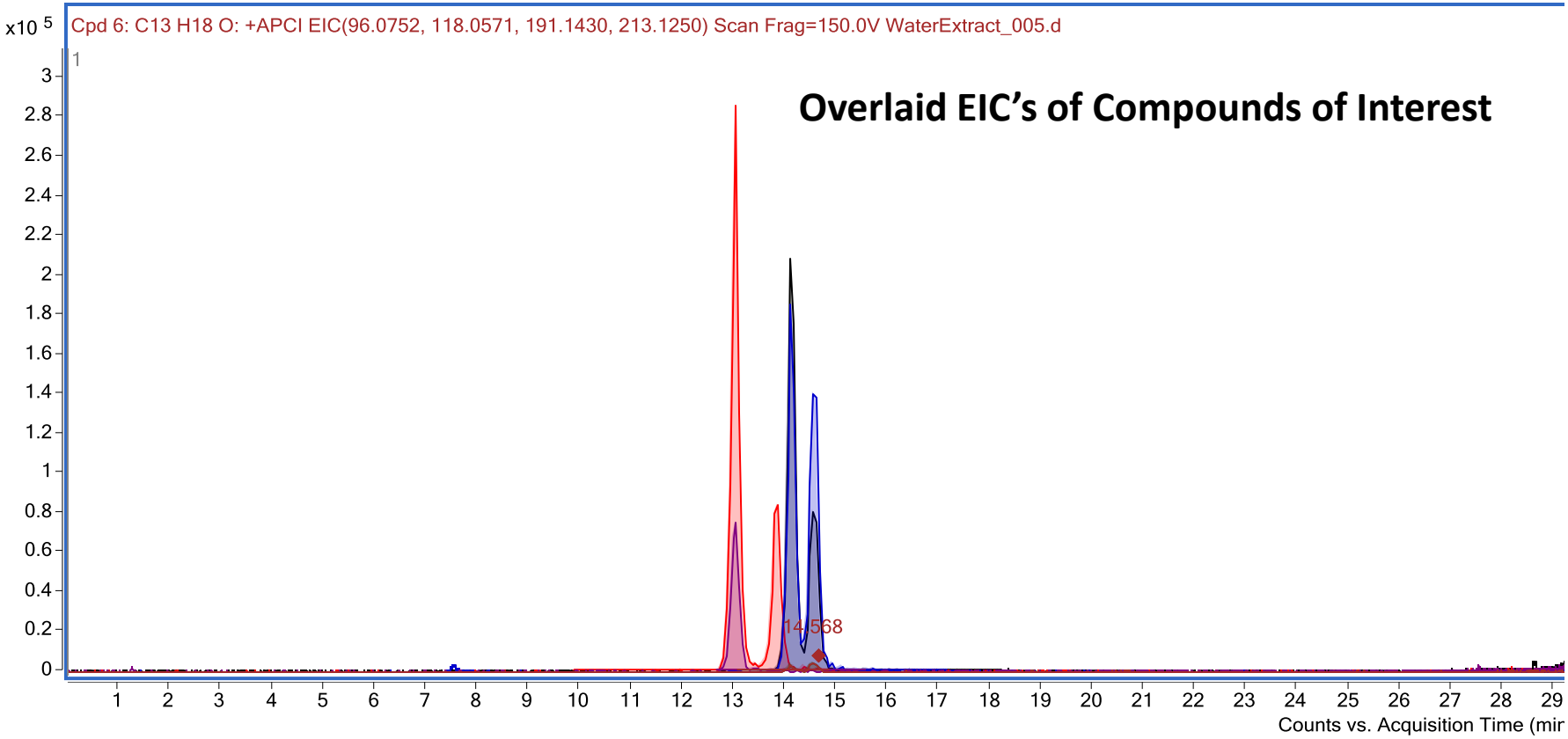
Qualitative Analysis: FBF



Qualitative Analysis: FBF



Qualitative Analysis FBF Results: Water Extracts



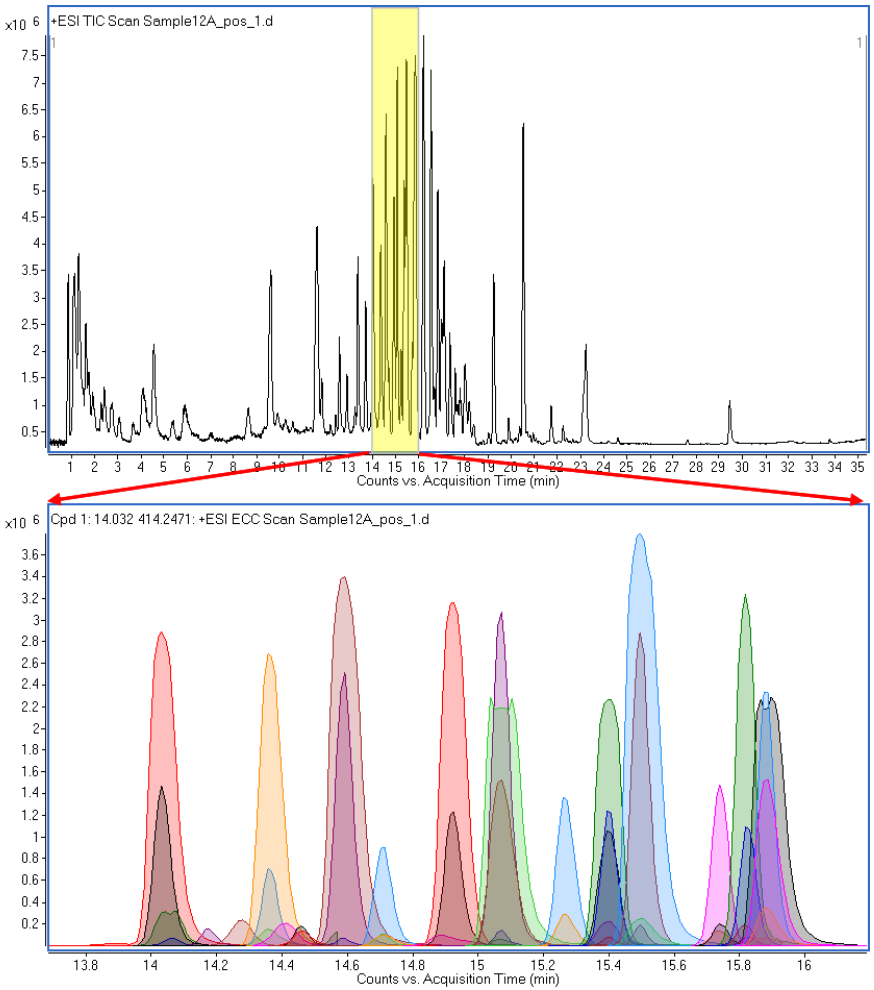
Compound List

Automatically Show Columns

Show/Hide	Cpd	File	Formula	RT	m/z	Mass	Score	Diff (T	Polarity	Height	Area	Algorithm	Flag Seve	Ions	ID Source
☑	1	WaterExtract_005.d	C12 H14	14.12	159.1168	158.1095	99.96	-0.16	Positive	185405	2016646	Find By Formula	Pass	3	FBF
☑	2	WaterExtract_005.d	C11 H12 O	13.057	161.0961	160.0889	99.32	0.7	Positive	285440	3080654	Find By Formula	Pass	3	FBF
☑	3	WaterExtract_005.d	C12 H16 O	14.558	177.1275	176.1202	99.73	0.55	Positive	81152	1097967	Find By Formula	Pass	3	FBF
☑	4	WaterExtract_005.d	C11 H14 O2	13.057	179.1067	178.0993	99.62	-0.19	Positive	75401	956647	Find By Formula	Pass	3	FBF
☑	5	WaterExtract_005.d	C13 H18 O	14.558	191.1427	190.1354	98.18	-1.79	Positive	3962	47566	Find By Formula	Pass	3	FBF

Approach 2: Molecular Feature Extractor (MFE)

The Workflow: Automated Data Mining Tools

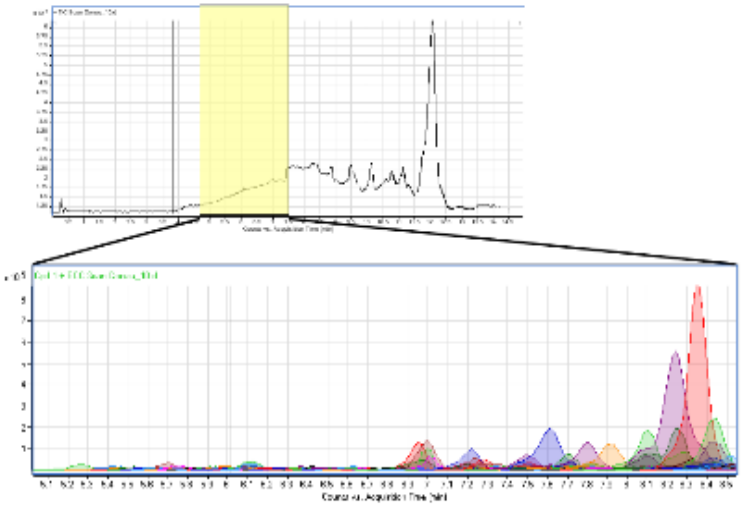


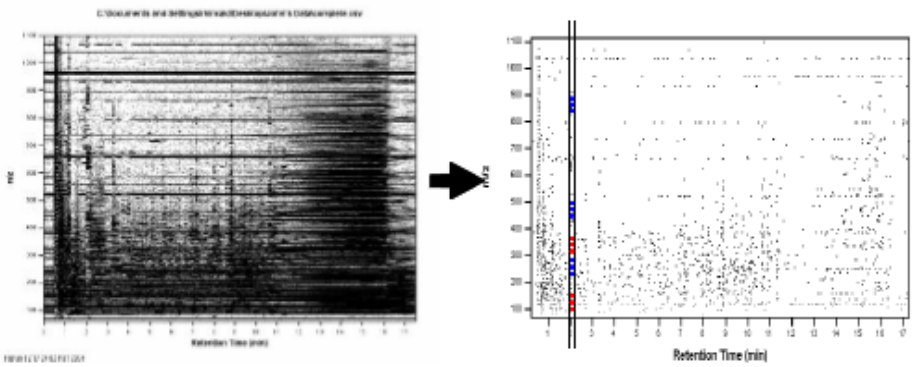
MassHunter Molecular Feature Extraction

Molecular Feature Extraction: Peak Finding Algorithm

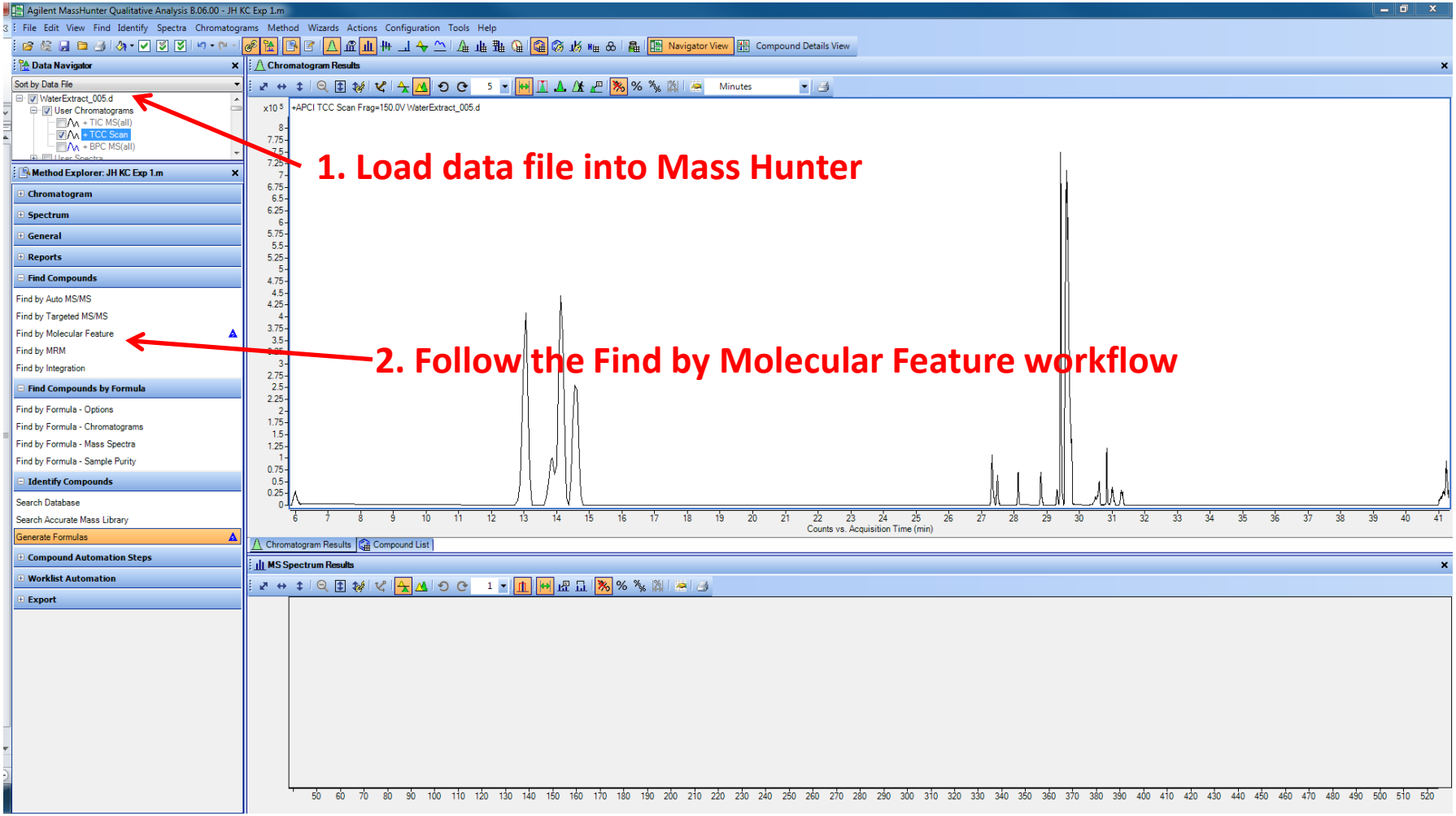
Data is processed by proprietary feature finding algorithm

- Find chromatographic peaks
  - Find all ions that are related
  - Include any adducts, such as Na^+ or K^+
  - Include isotopes ($[M+H]^+$, $[M+H+1]^+$,…
  - Check for dimers
  - Create a compound chromatograms (ECC) and spectra
- Sum all ion signals into one value (Feature)
- Fully automated processing
- Create data file for export

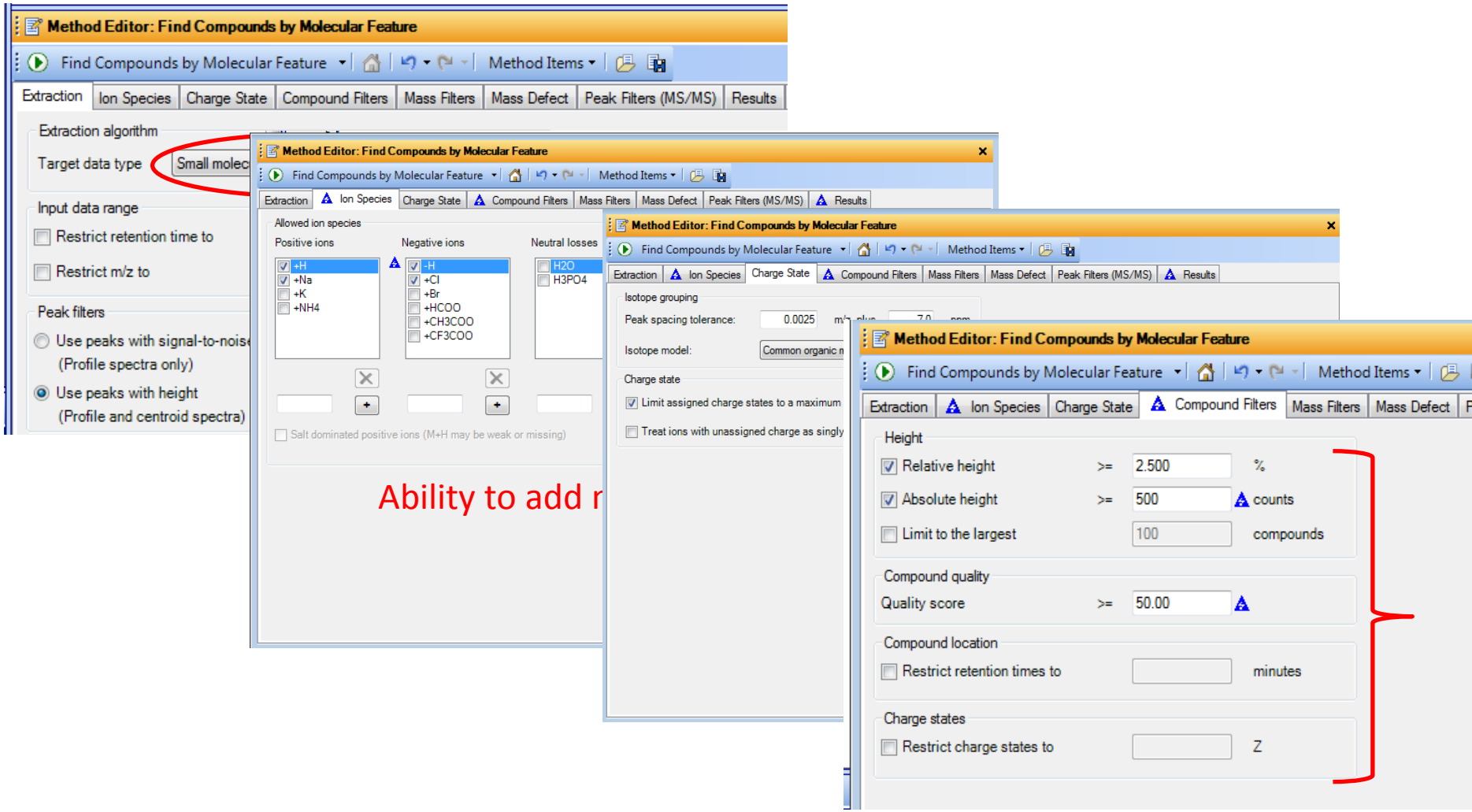




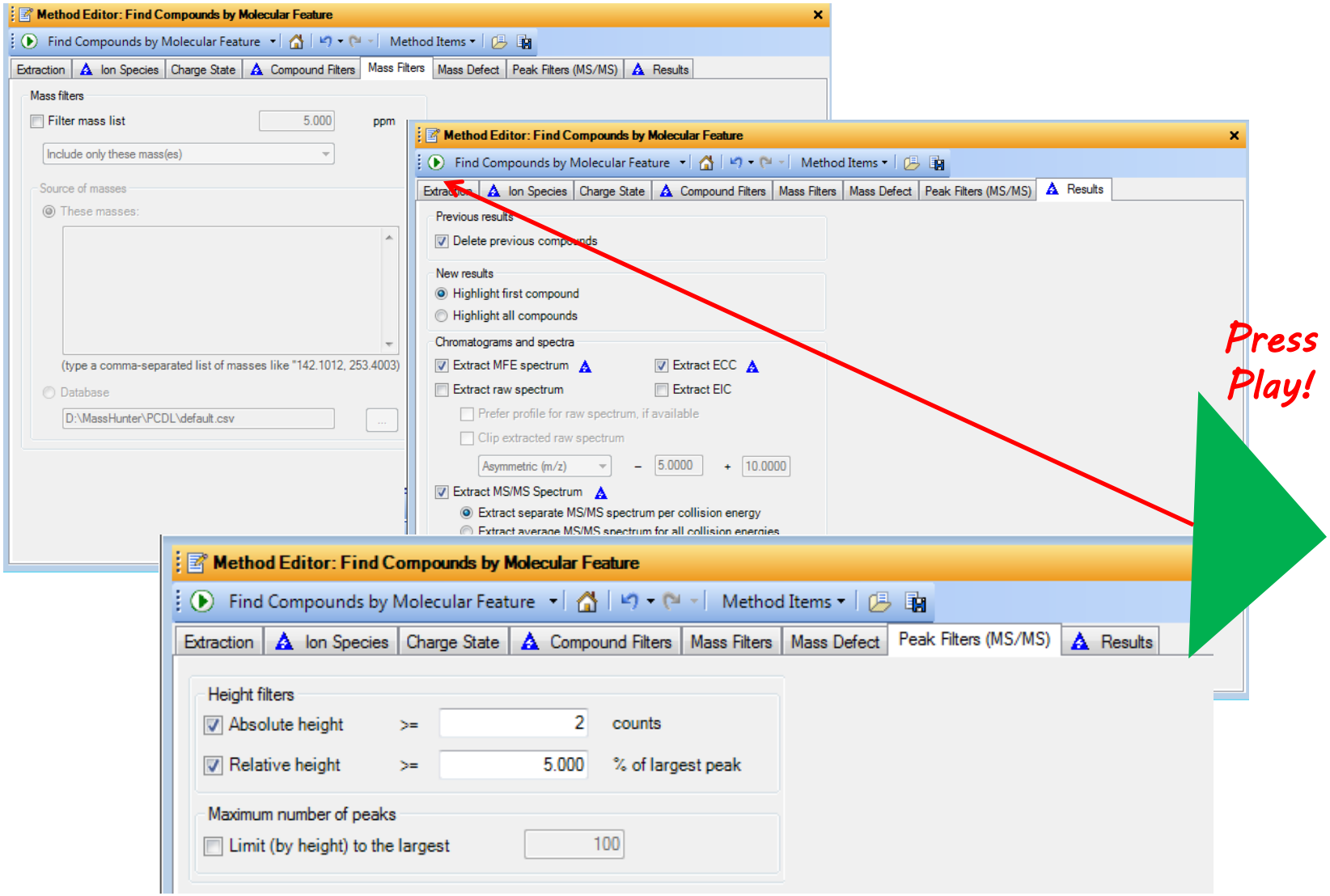
Qualitative Analysis Water Extracts: MFE



Qualitative Analysis Water Extracts: MFE

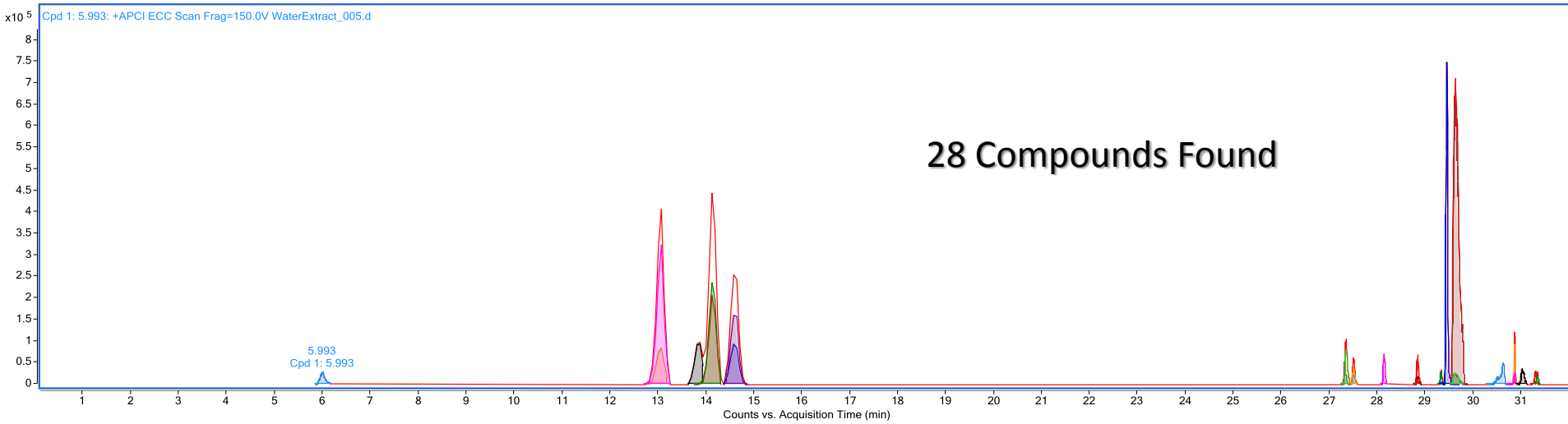


Qualitative Analysis Water Extracts: MFE



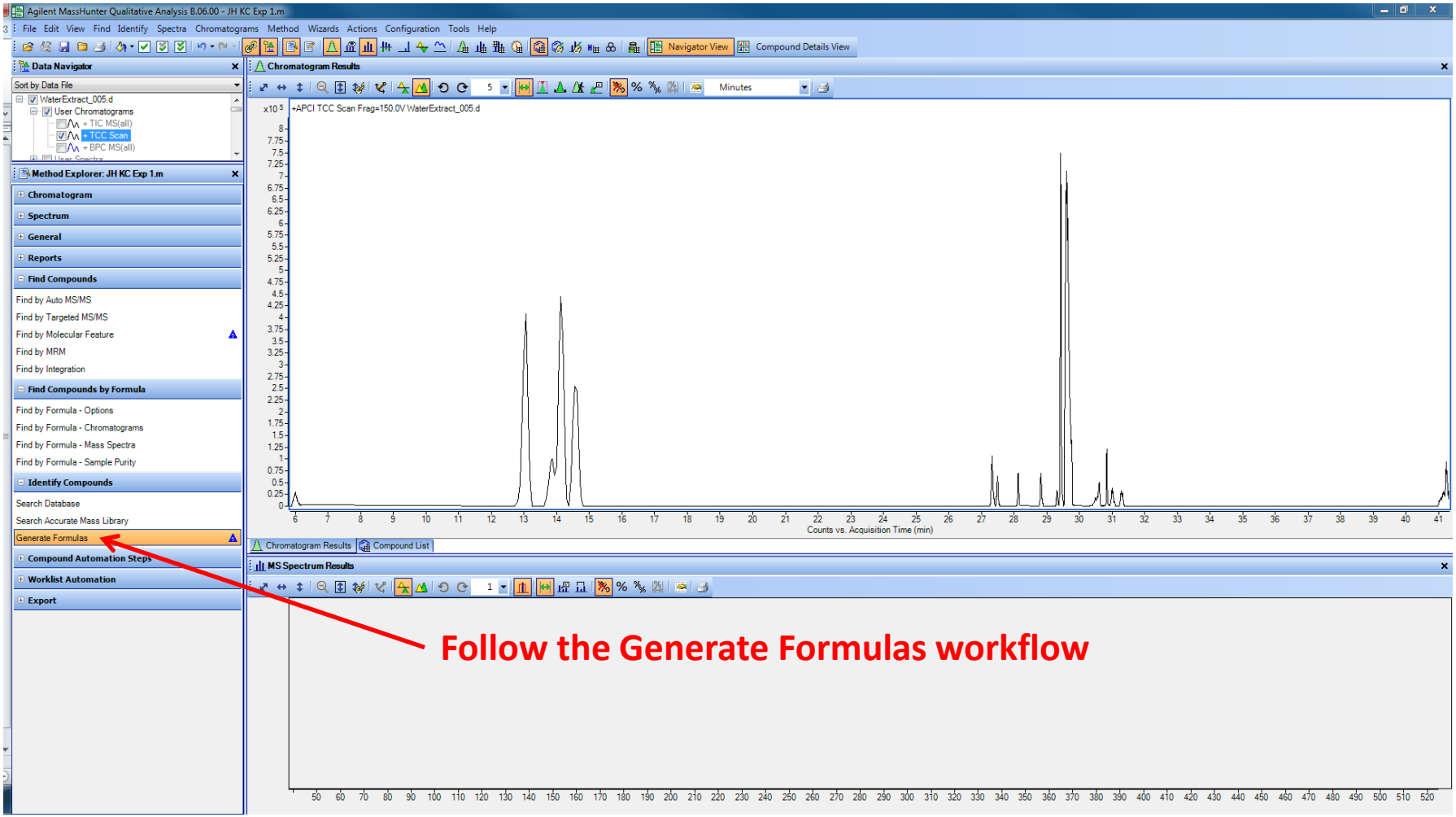
Qualitative Analysis MFE Results: Water Extracts

Overlaid Extracted Compound Chromatograms of Compounds resulting from the parameters set in MFE peak picking algorithm

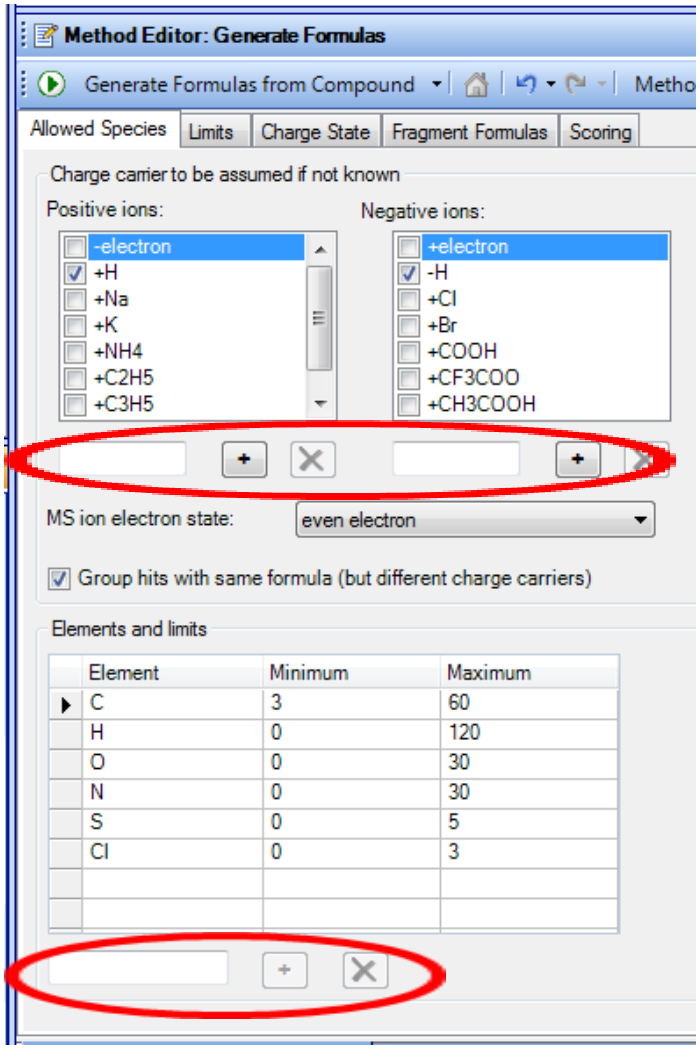


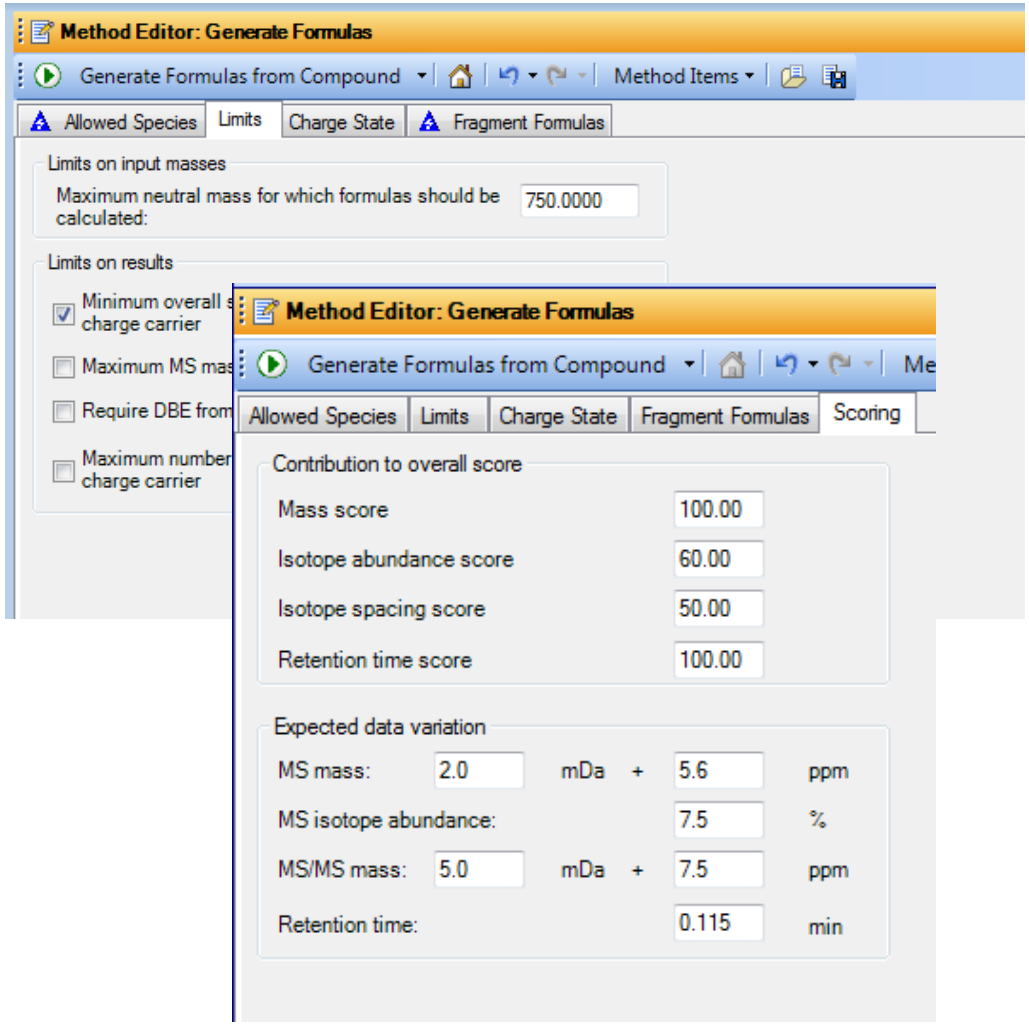
Next step: Generate formulae from compounds

Qualitative Analysis: Generate Formulae from MFE Results

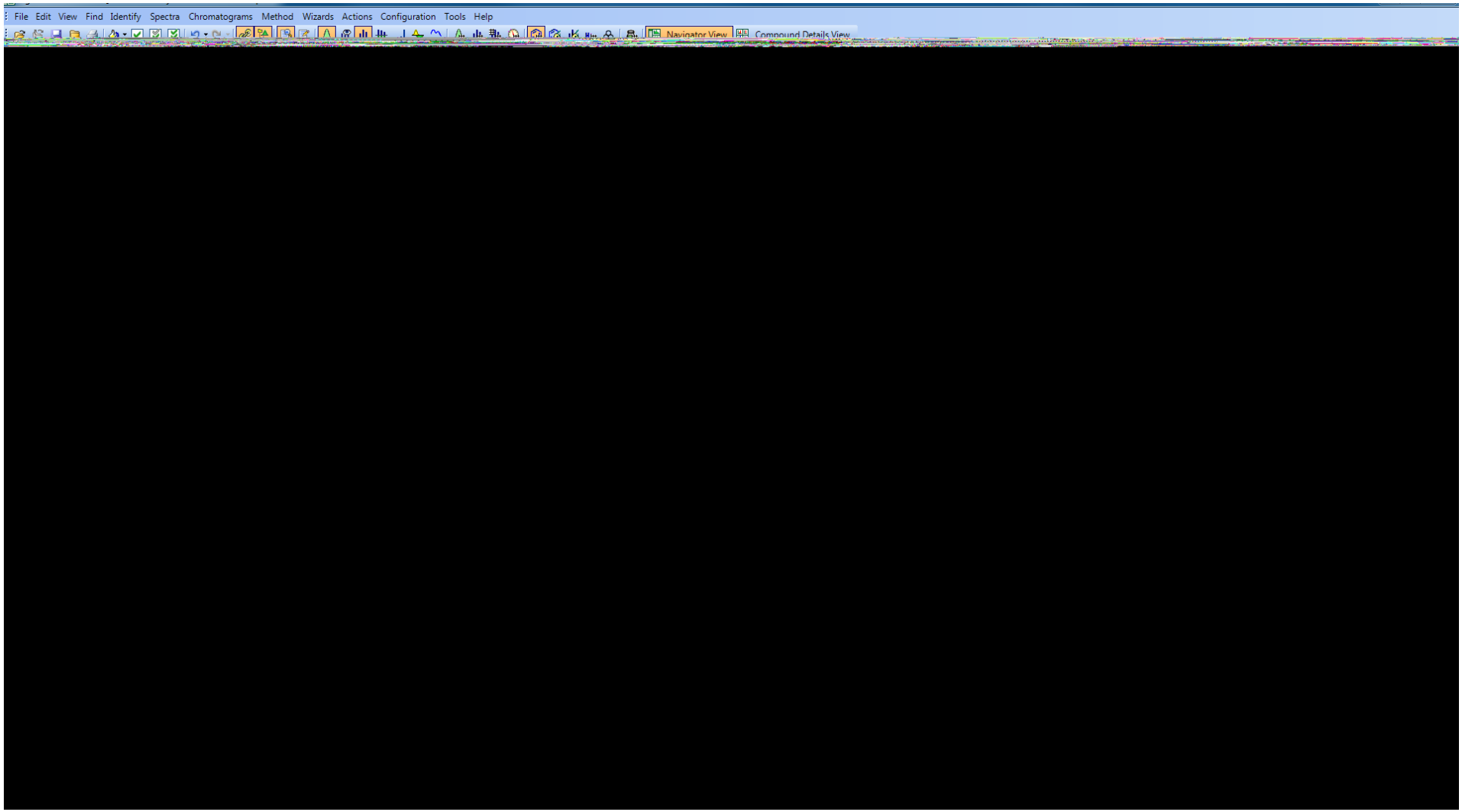


Qualitative Analysis: Generate Formulae from MFE Results

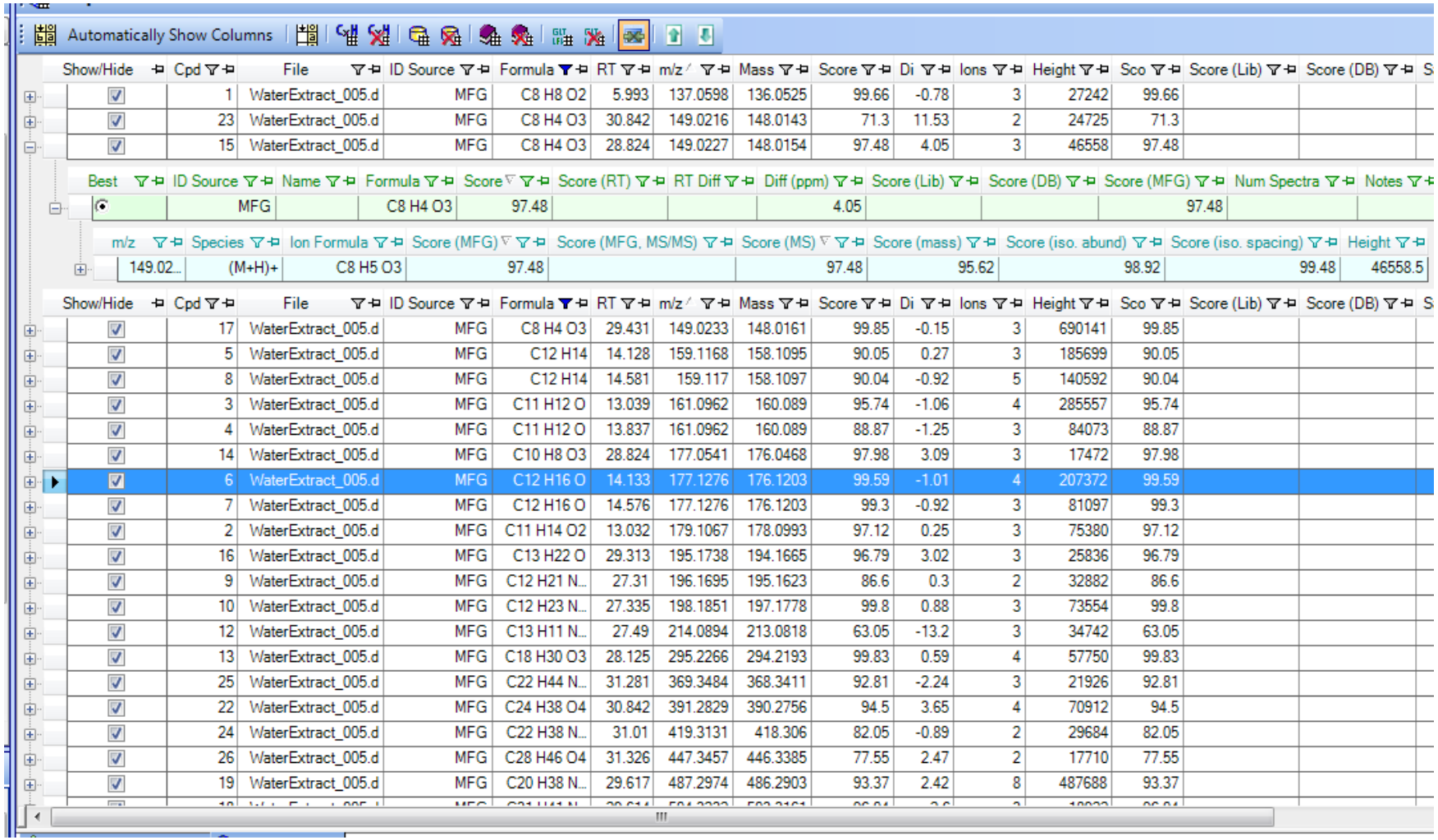




Qualitative Analysis: Formula Generation Results



Qualitative Analysis: Formula Generation Results



Automatically Show Columns

Show/Hide	Cpd	File	ID Source	Formula	RT	m/z	Mass	Score	Di	Ions	Height	Sco	Score (Lib)	Score (DB)
☑	1	WaterExtract_005.d	MFG	C8 H8 O2	5.993	137.0598	136.0525	99.66	-0.78	3	27242	99.66		
☑	23	WaterExtract_005.d	MFG	C8 H4 O3	30.842	149.0216	148.0143	71.3	11.53	2	24725	71.3		
☑	15	WaterExtract_005.d	MFG	C8 H4 O3	28.824	149.0227	148.0154	97.48	4.05	3	46558	97.48		

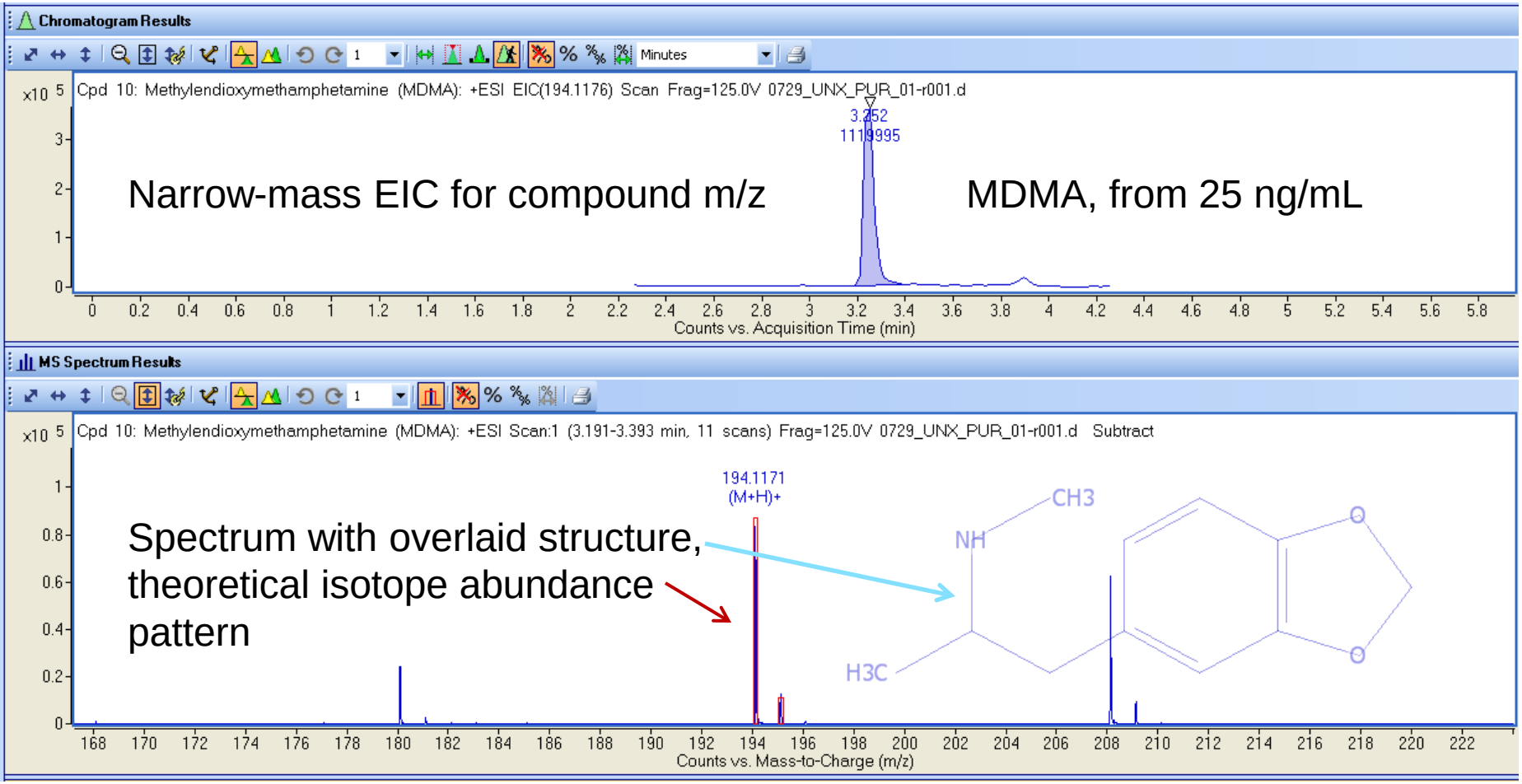
Best	ID Source	Name	Formula	Score	Score (RT)	RT Diff	Diff (ppm)	Score (Lib)	Score (DB)	Score (MFG)	Num Spectra	Notes
◉	MFG		C8 H4 O3	97.48			4.05			97.48		

m/z	Species	Ion Formula	Score (MFG)	Score (MFG, MS/MS)	Score (MS)	Score (mass)	Score (iso. abund)	Score (iso. spacing)	Height
149.02...	(M+H)+	C8 H5 O3	97.48		97.48	95.62	98.92	99.48	46558.5

Show/Hide	Cpd	File	ID Source	Formula	RT	m/z	Mass	Score	Di	Ions	Height	Sco	Score (Lib)	Score (DB)
☑	17	WaterExtract_005.d	MFG	C8 H4 O3	29.431	149.0233	148.0161	99.85	-0.15	3	690141	99.85		
☑	5	WaterExtract_005.d	MFG	C12 H14	14.128	159.1168	158.1095	90.05	0.27	3	185699	90.05		
☑	8	WaterExtract_005.d	MFG	C12 H14	14.581	159.117	158.1097	90.04	-0.92	5	140592	90.04		
☑	3	WaterExtract_005.d	MFG	C11 H12 O	13.039	161.0962	160.089	95.74	-1.06	4	285557	95.74		
☑	4	WaterExtract_005.d	MFG	C11 H12 O	13.837	161.0962	160.089	88.87	-1.25	3	84073	88.87		
☑	14	WaterExtract_005.d	MFG	C10 H8 O3	28.824	177.0541	176.0468	97.98	3.09	3	17472	97.98		
☑	6	WaterExtract_005.d	MFG	C12 H16 O	14.133	177.1276	176.1203	99.59	-1.01	4	207372	99.59		
☑	7	WaterExtract_005.d	MFG	C12 H16 O	14.576	177.1276	176.1203	99.3	-0.92	3	81097	99.3		
☑	2	WaterExtract_005.d	MFG	C11 H14 O2	13.032	179.1067	178.0993	97.12	0.25	3	75380	97.12		
☑	16	WaterExtract_005.d	MFG	C13 H22 O	29.313	195.1738	194.1665	96.79	3.02	3	25836	96.79		
☑	9	WaterExtract_005.d	MFG	C12 H21 N...	27.31	196.1695	195.1623	86.6	0.3	2	32882	86.6		
☑	10	WaterExtract_005.d	MFG	C12 H23 N...	27.335	198.1851	197.1778	99.8	0.88	3	73554	99.8		
☑	12	WaterExtract_005.d	MFG	C13 H11 N...	27.49	214.0894	213.0818	63.05	-13.2	3	34742	63.05		
☑	13	WaterExtract_005.d	MFG	C18 H30 O3	28.125	295.2266	294.2193	99.83	0.59	4	57750	99.83		
☑	25	WaterExtract_005.d	MFG	C22 H44 N...	31.281	369.3484	368.3411	92.81	-2.24	3	21926	92.81		
☑	22	WaterExtract_005.d	MFG	C24 H38 O4	30.842	391.2829	390.2756	94.5	3.65	4	70912	94.5		
☑	24	WaterExtract_005.d	MFG	C22 H38 N...	31.01	419.3131	418.306	82.05	-0.89	2	29684	82.05		
☑	26	WaterExtract_005.d	MFG	C28 H46 O4	31.326	447.3457	446.3385	77.55	2.47	2	17710	77.55		
☑	19	WaterExtract_005.d	MFG	C20 H38 N...	29.617	487.2974	486.2903	93.37	2.42	8	487688	93.37		

Identifies isomers as separate entities due to different retention times

Typical Target Compound Screening Result

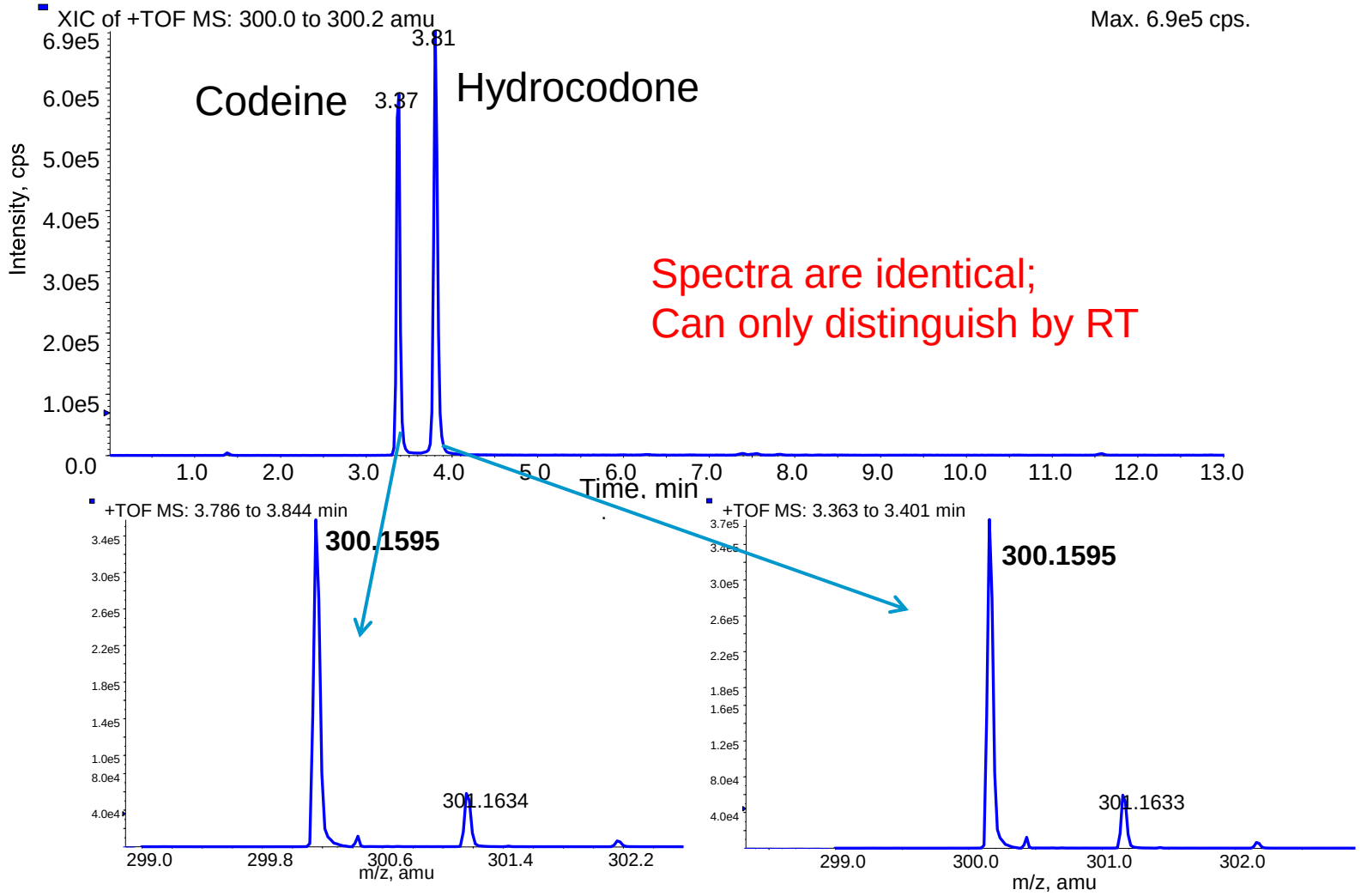


Text Result and Figures of Merit

DB Search Results: Cpd 10: Methylendioxymethamphetamine (MDMA)

Best	Name	Formula	Mass	Mass (DB)	Diff (ppm)	RT	RT Diff	RT Match	Mass Match	Spacing Match	Abund Match	Score
✓	Methylendioxymethamphetamine (MDMA)	C11 H15 N O2	193.1099	193.1103	2.16	3.252	0.006	99.61	98.19	10	91.82	83.19

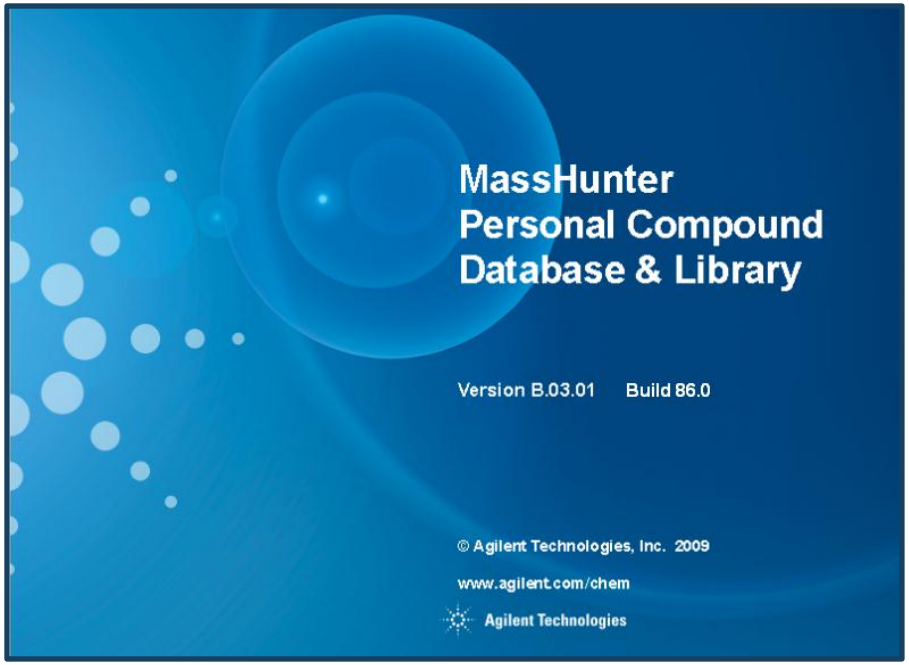
Chromatography still matters: Identification of isobaric compounds $C_{18}H_{21}NO_3$



Agilent Technologies

Personal Compound Database (PCDL)

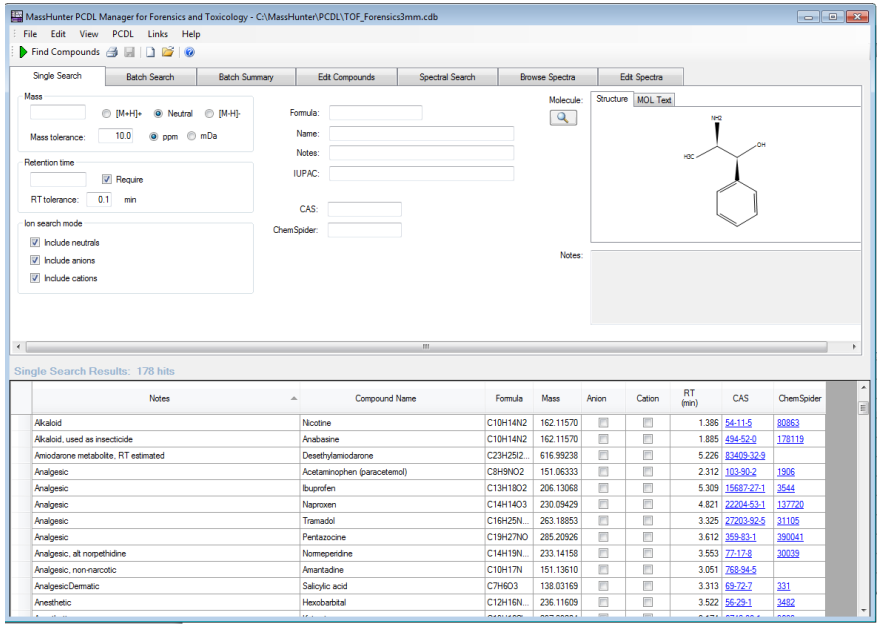
- Fully integrated Agilent solution
- METLIN Metabolite-specific database content
- Contains over 25,000 compounds
- Includes 8,000 lipids
- Manual and batch searches
- Query based on monoisotopic mass, formula, name or retention time
- Build customized database using your own compounds and assign retention time

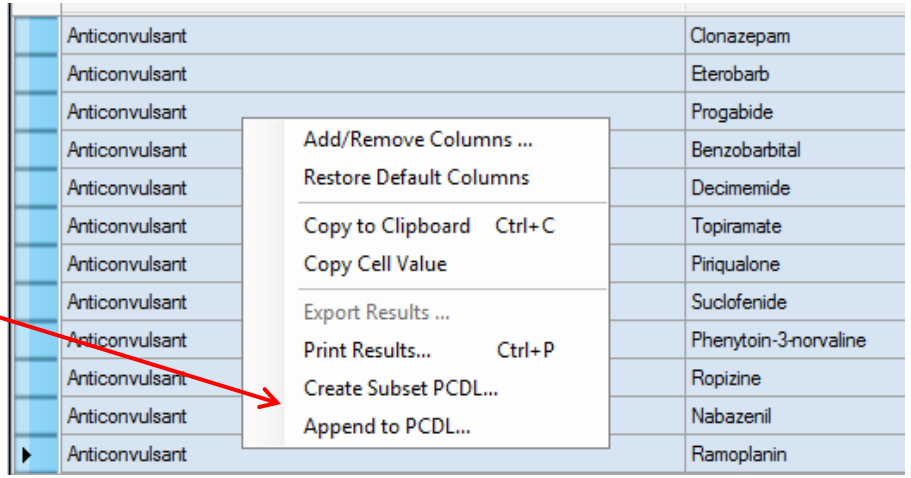


Optional accurate mass & retention time (AMRT) database based on the analysis of hundreds of standards

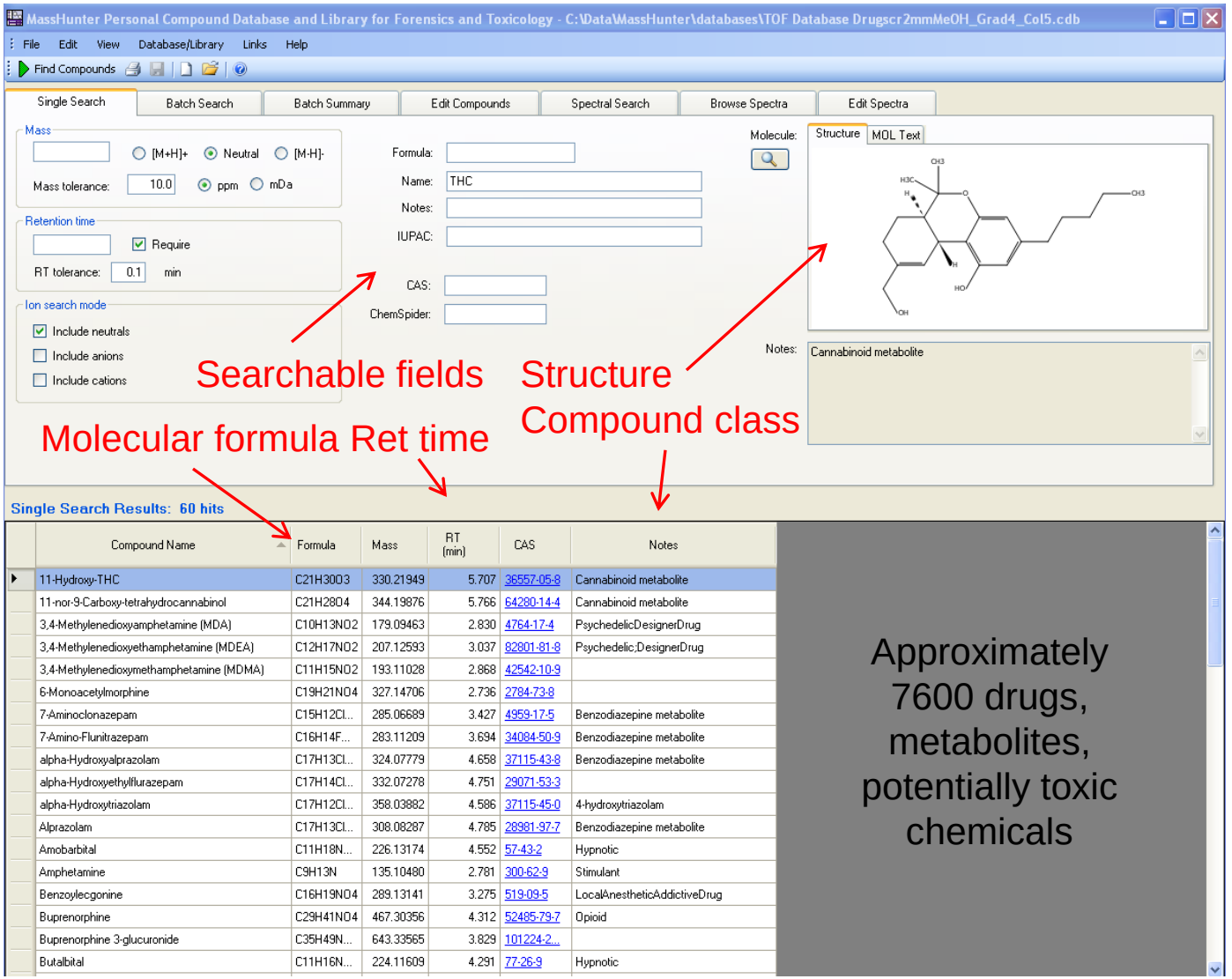
PCDL Manager B.04

- Newest version automatically converts previous PCDL's to B.04 format, saves original as [filename].cdb.bk backup
- Includes ability to use Agilent QTOF MS/MS PCDL database/libraries, or to add user spectra to custom cdb.
- Easier to make custom databases with "Create subset PCDL"
  1. Search and select entries
  2. R-click in listing,
  select Create (to make new cdb) or Append to (existing) PCDL.

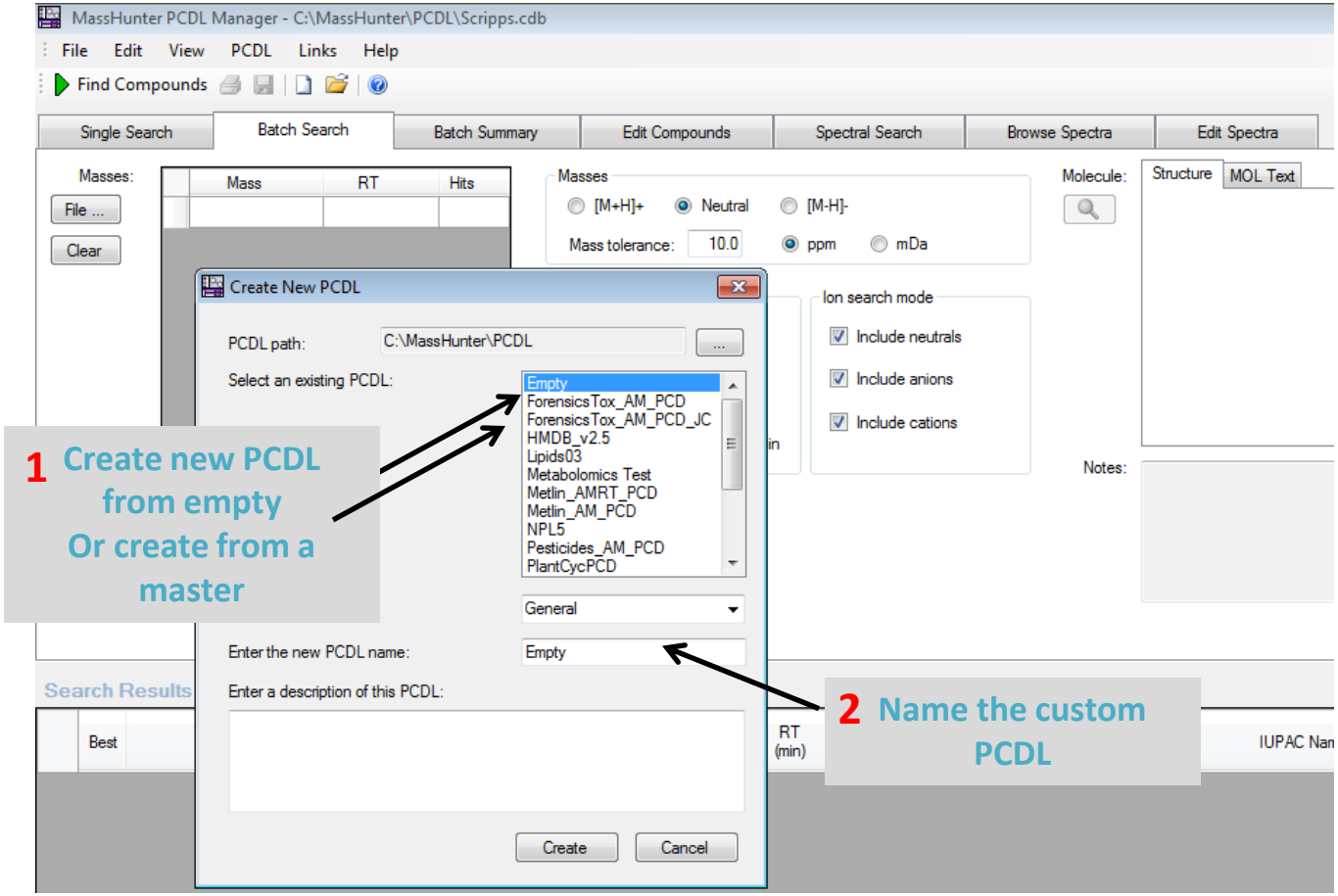




MassHunter PCDL: AMRT Database for Forensics

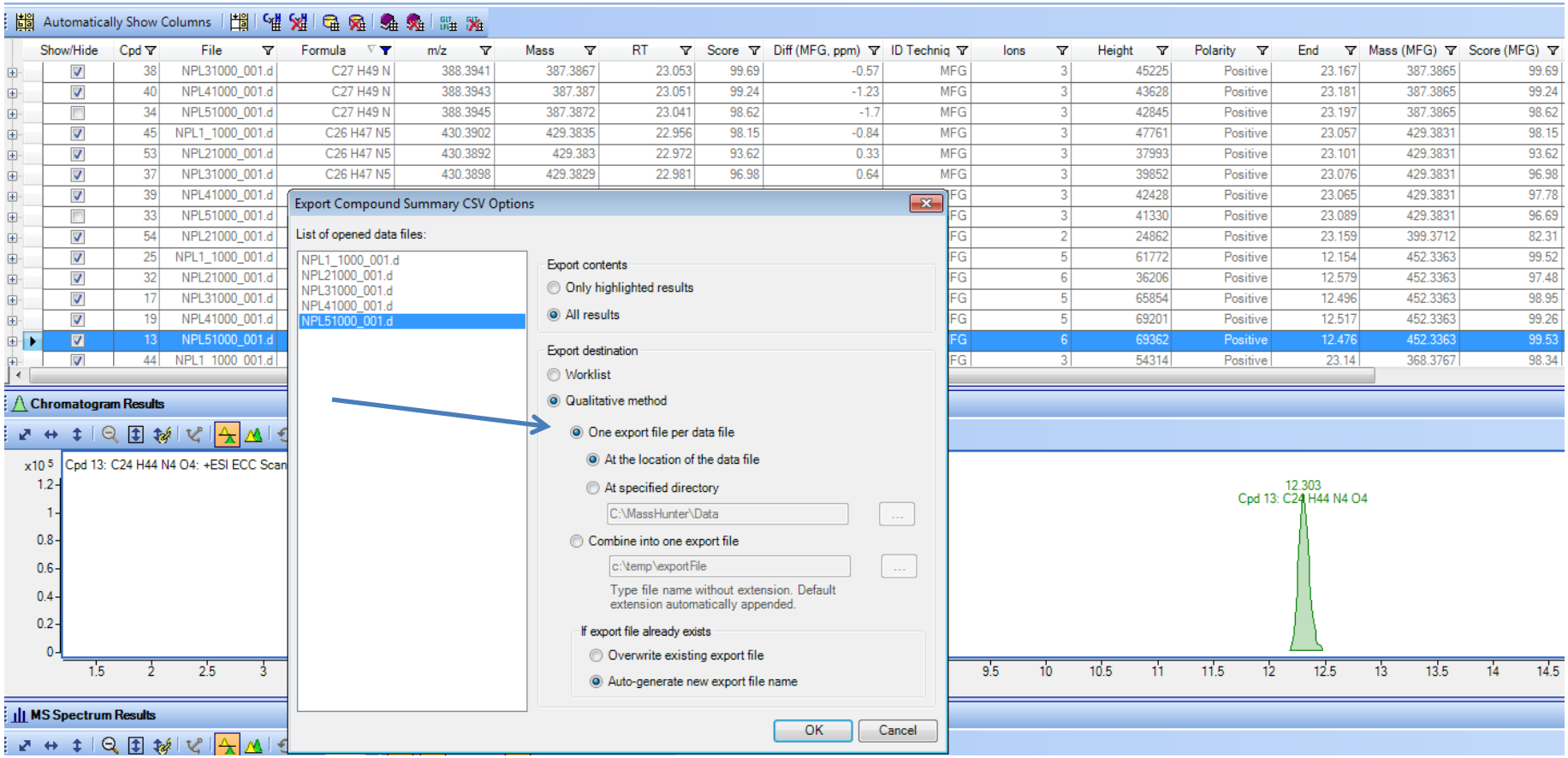


Create Custom PCDL



Export Compound List to Excel

After data analysis using Find by Formula with Database search or MFE / MFG



Template for Import into PCDL

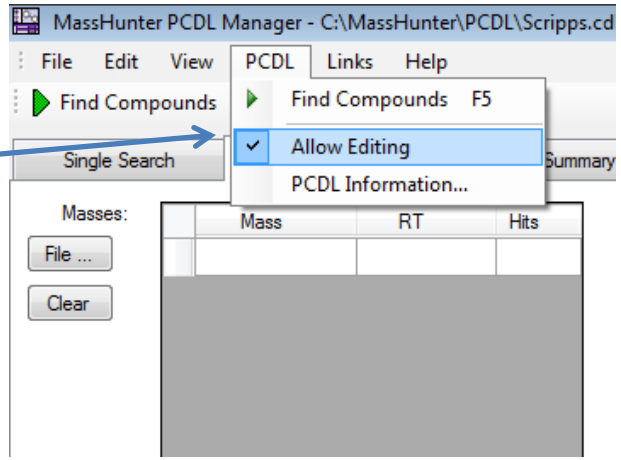
Insert the exported data into a template formatted for importing into PCDL Not intuitive…

Note: PCDL contains mass not m/z!

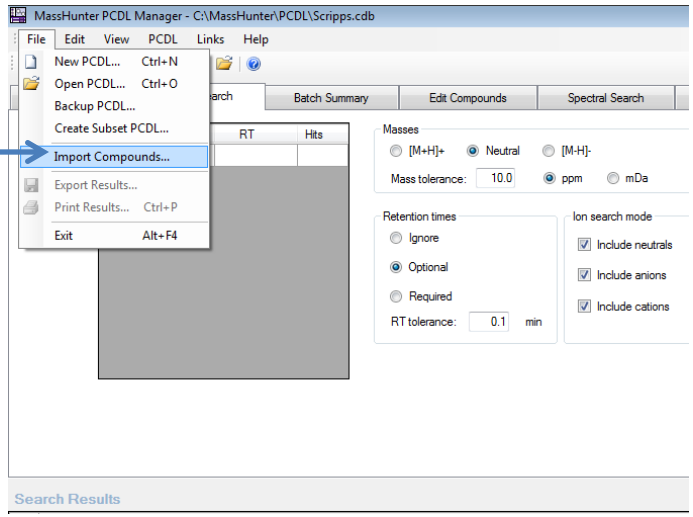
Compounds					
Formula	Retention	Mass	Compoun	CAS Notes	
C20 H42 O	10.303	458.2728	12		
C24 H44 N	12.303	452.3363	13		
C26 H47 N	22.967	429.384	33		
C27 H49 N	23.041	387.3872	34		
C11 H26 N	21.828	378.1481	27		
C16 H34 O	9.116	370.2207	10		
C23 H48 N	22.909	368.3776	32		
C14 H26 O	8.68	354.1528	9		
C14 H30 O	8.383	326.1942	8		
C20 H10 N	20.354	326.0712	22		
C18 H29 N	21.829	323.2101	29		
C18 H18 O	19.193	314.1157	20		
C19 H20 O	21.64	312.1367	25		
C12 H22 O	7.845	310.1268	7		
C12 H24 O	7.68	296.1475	6		
C19 H41 N	21.593	283.3243	24		
C12 H26 O	7.524	282.1683	5		
C16 H22 O	21.83	278.1526	30		
C16 H22 O	21.585	278.1523	23		
C12 H12 N	17.43	272.103	18		
C10 H18 O	6.805	266.1004	4		
C13 H16 N	22.577	256.1449	31		
C10 H20 O	6.634	252.1211	3		
C14 H18 O	19.46	250.1208	21		
C11 H12 N	17.364	244.1073	17		
C10 H8 N6	16.799	244.072	16		
C10 H22 O	6.484	238.1418	2		
C14 H31 N	18.764	229.2408	19		
C12 H12 O	21.828	204.0791	26		
C9 H18 O4	14.647	190.1207	15		
C8 H18 O3	12.931	162.1257	14		
C8 H4 O3	21.828	148.0163	28		

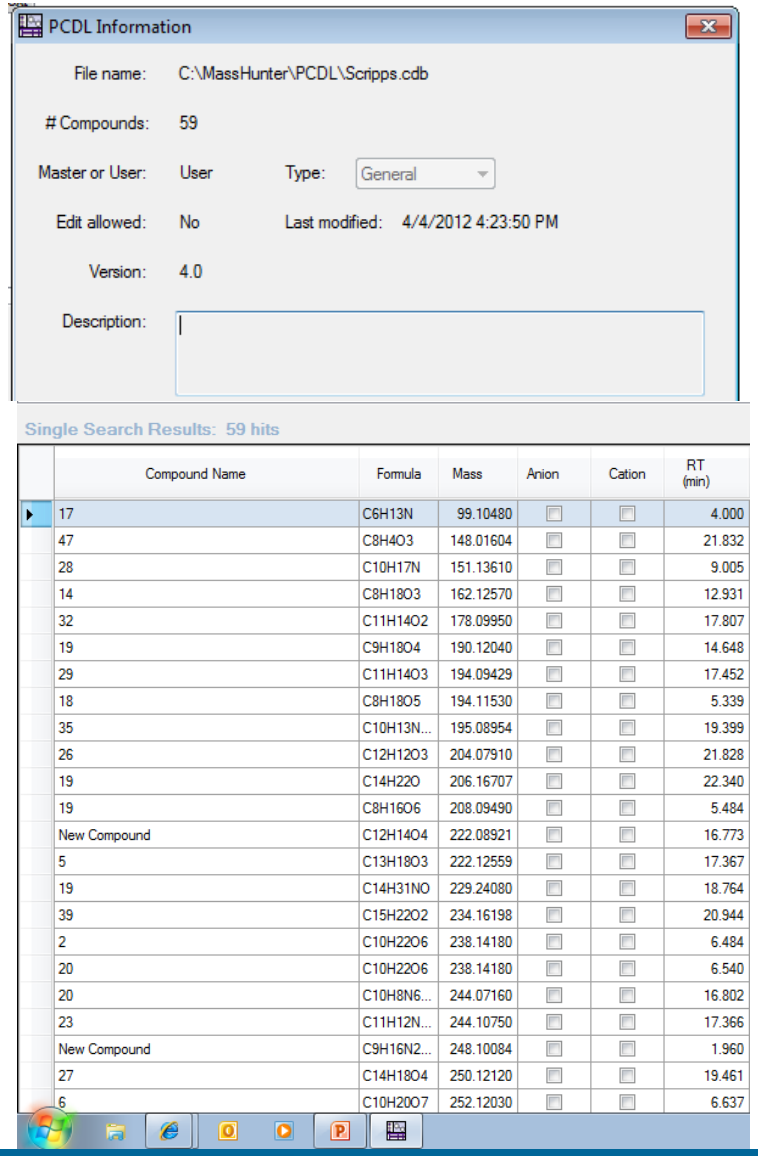
Import Compounds into PCDL

3



4



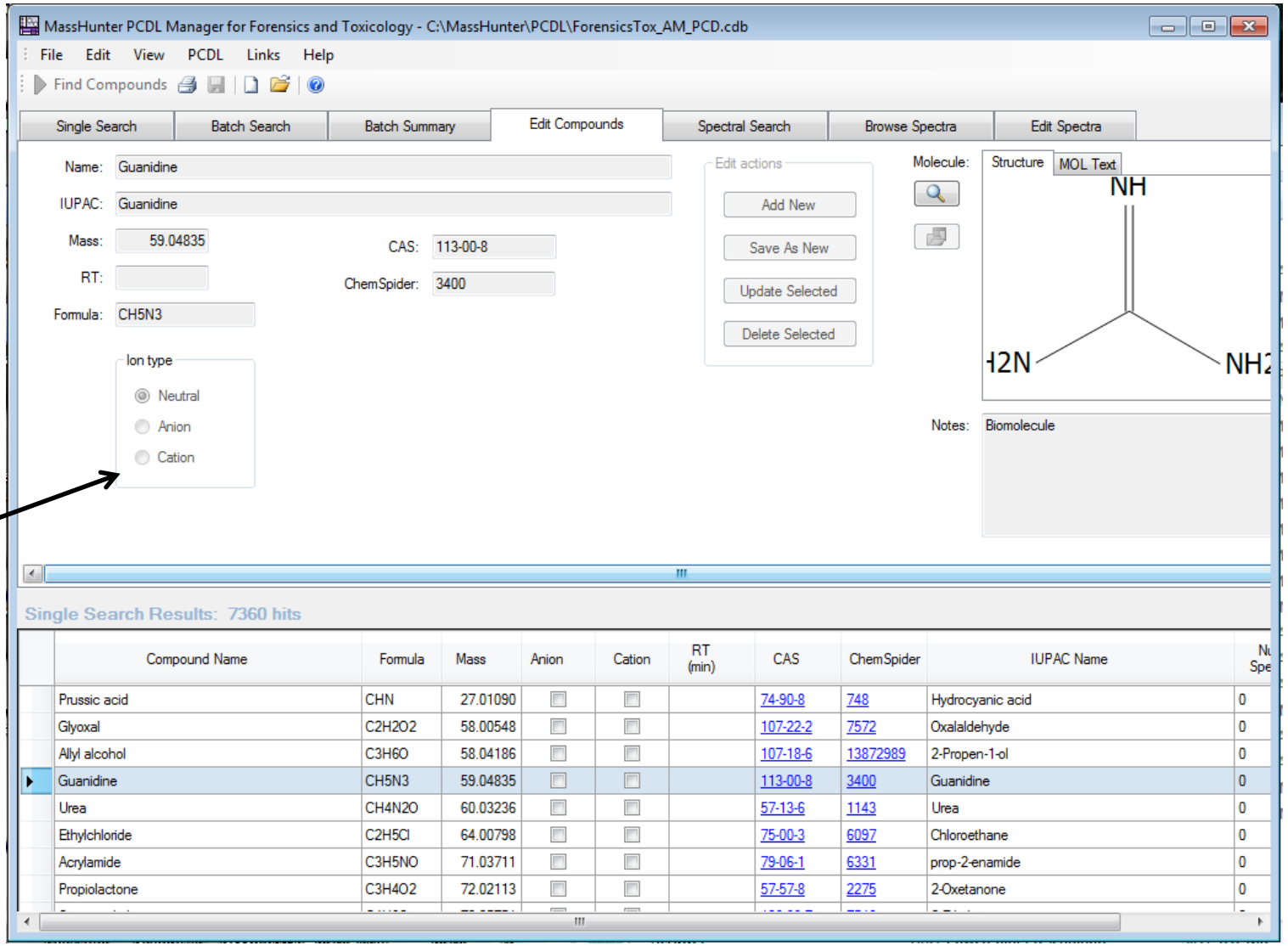


Compound Name	Formula	Mass	Anion	Cation	RT (min)
17	C6H13N	99.10480			4.000
47	C8H4O3	148.01604			21.832
28	C10H17N	151.13610			9.005
14	C8H18O3	162.12570			12.931
32	C11H14O2	178.09950			17.807
19	C9H18O4	190.12040			14.648
29	C11H14O3	194.09429			17.452
18	C8H18O5	194.11530			5.339
35	C10H13N...	195.08954			19.399
26	C12H12O3	204.07910			21.828
19	C14H22O	206.16707			22.340
19	C8H16O6	208.09490			5.484
New Compound	C12H14O4	222.08921			16.773
5	C13H18O3	222.12559			17.367
19	C14H31NO	229.24080			18.764
39	C15H22O2	234.16198			20.944
2	C10H22O6	238.14180			6.484
20	C10H22O6	238.14180			6.540
20	C10H8N6...	244.07160			16.802
23	C11H12N...	244.10750			17.366
New Compound	C9H16N2...	248.10084			1.960
27	C14H18O4	250.12120			19.461
6	C10H20O7	252.12030			6.637

View and Edit Compounds in PCDL

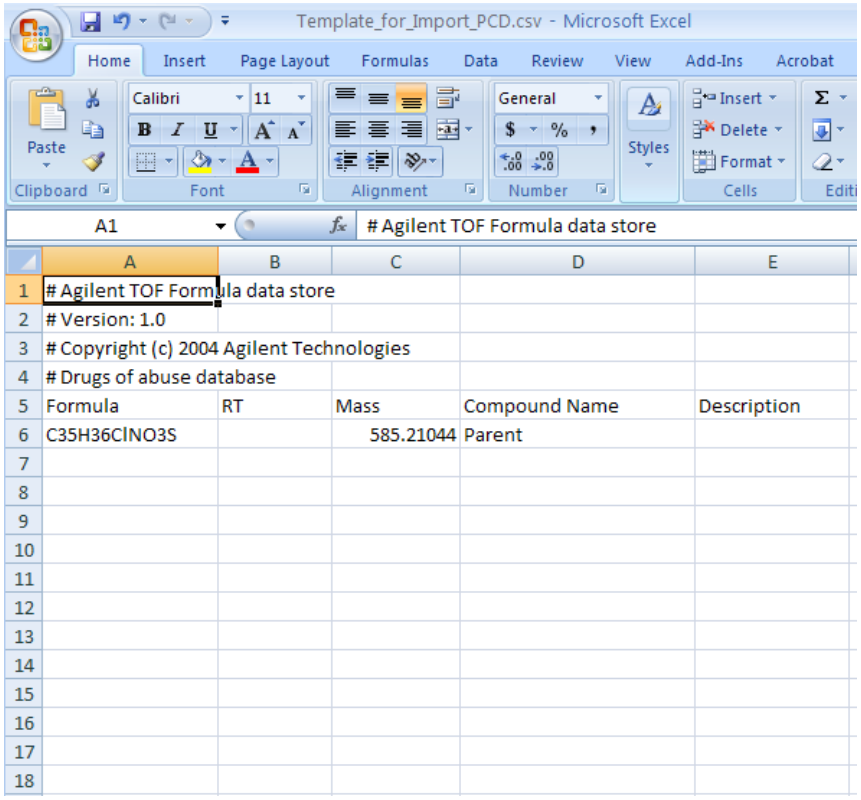
!!! If quaternary salt it must be noted as such !!!

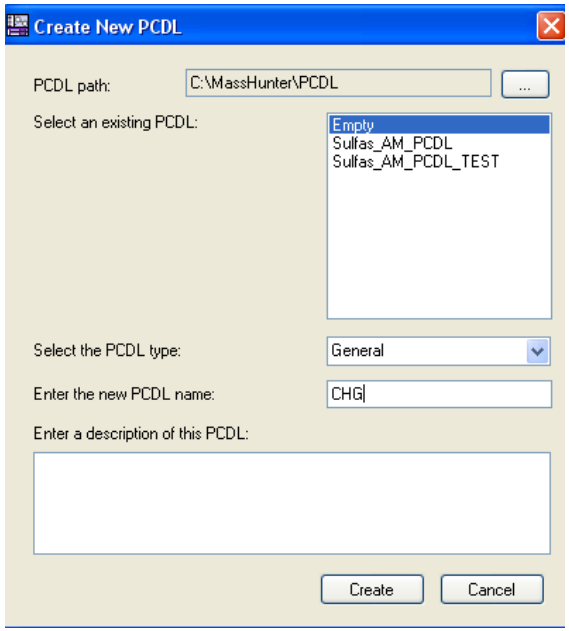
Click cation radio button to make this identification

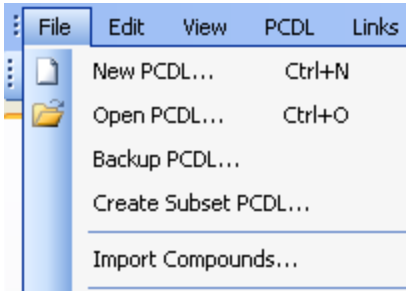


Compound Name	Formula	Mass	Anion	Cation	RT (min)	CAS	ChemSpider	IUPAC Name	Nu Spe
Prussic acid	CHN	27.01090				74-90-8	748	Hydrocyanic acid	0
Glyoxal	C2H2O2	58.00548				107-22-2	7572	Oxalaldehyde	0
Allyl alcohol	C3H6O	58.04186				107-18-6	13872989	2-Propen-1-ol	0
Guanidine	CH5N3	59.04835				113-00-8	3400	Guanidine	0
Urea	CH4N2O	60.03236				57-13-6	1143	Urea	0
Ethylchloride	C2H5Cl	64.00798				75-00-3	6097	Chloroethane	0
Acrylamide	C3H5NO	71.03711				79-06-1	6331	prop-2-enamide	0
Propiolactone	C3H4O2	72.02113				57-57-8	2275	2-Oxetanone	0

Personal Databases Cut/Paste to Template







Import Compounds into New PCDL Database

Frequently Asked Questions

1. *What criteria do labs typically use to call a positive using this methodology?*
   Typical criteria are: Score ≥ 70, mass error ≤ 12 ppm, RT difference ≤ 0.12 min

   Crashed serum has good hits that can have Scores in the 50-70 range due to chemical interferences with the isotopes. You can lower the effect of isotopes using Match Scoring section of the Qual method, but you are just artificially adjusting your Scores upwards by doing that. We have seen mass errors > 15 ppm for a few true positive compounds in particular samples.

   Labs with better sample cleanup (SPE, LLE, TOXI-TUBES) typically have Scores in the 80-90 range and mass errors < 10 ppm.

   Typical RT differences are 0.02-0.05 minutes. Values larger than those lower the Score, call the identification into question, or may indicate an LC or mobile phase problem. The RT Match Tolerance in the method must be sufficiently small to differentiate any close-eluting isobars such as codeine/hydrocodone (UT State Health Dept uses 0.1min rather than 0.12)

   Part of your method validation must include:
   - establishing what the positive identification criteria will be for your SOP.

   - establishing the variation in Score, mass error and RT difference for replicate preps on multiple days at the cutoff and 50% of the cutoff for each drug. Running fortified samples at 50% of the desired cutoff will help establish your identification criteria and something like an LOD for this screen.

Frequently Asked Questions

2. Can you somehow import Qual results into MassHunter Quant to facilitate accurate-mass Quant method setup?

The most important feature is importing FbF compound information (name, EIC m/z, RT) from results exported as a .cef file (rather than .csv). This takes the place of typing in information into Quant for each compound.

This release had not been sufficiently tested or documented to cover its use during this meeting. The export/import process and new Quant features will be covered during the next TOF Drugscreen User meeting in (approximately) February 2013.