Agilent 6400 Series Triple Quadrupole LC/MS/MS Users Session

QQQ Method Development and Optimization

MassHunter Quant:

Method setup

Peak detection optimization

Quant troubleshooting

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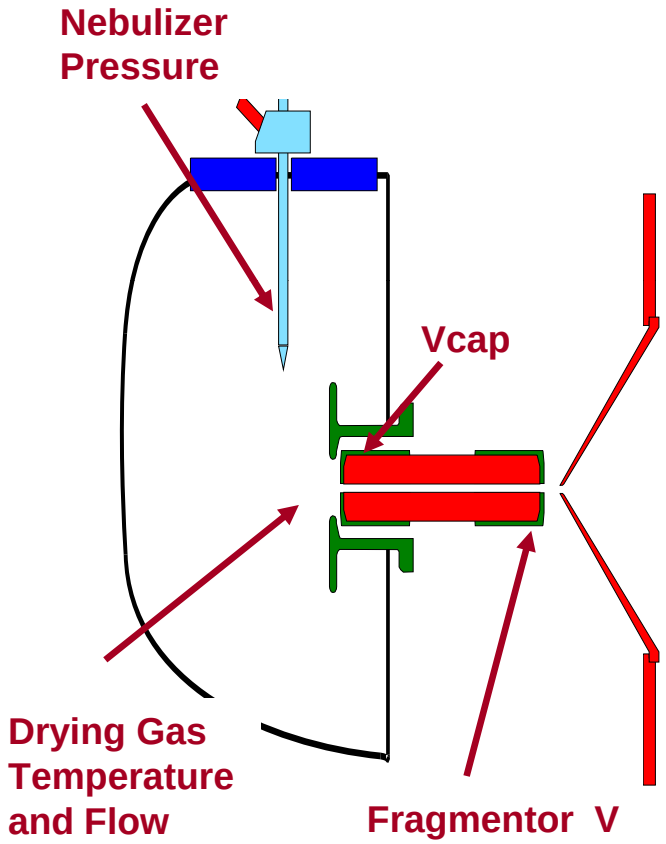
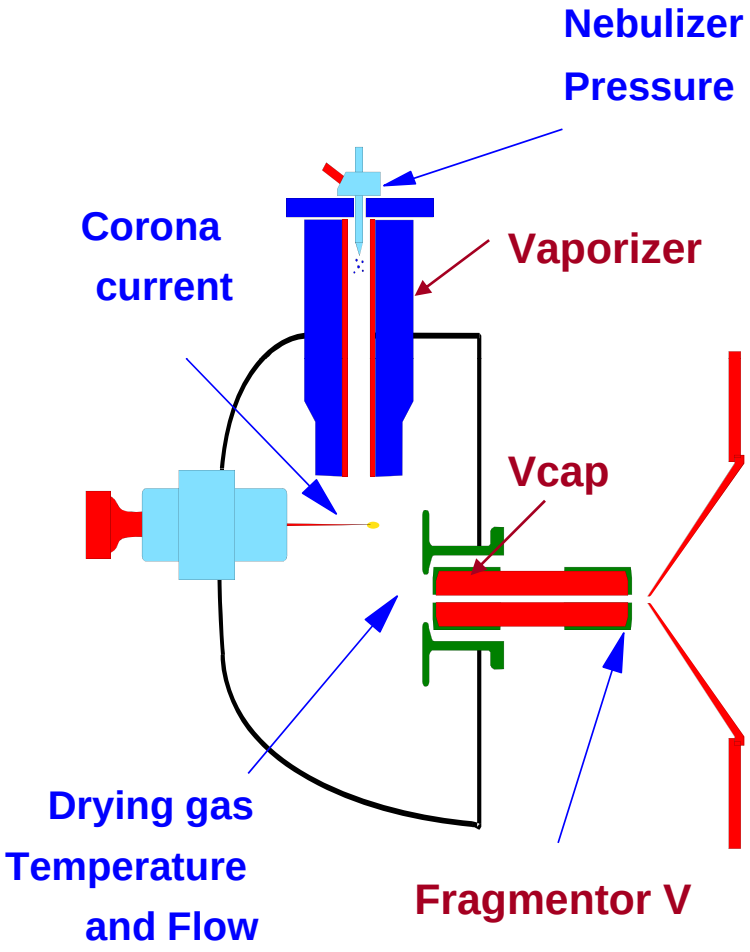
Agenda

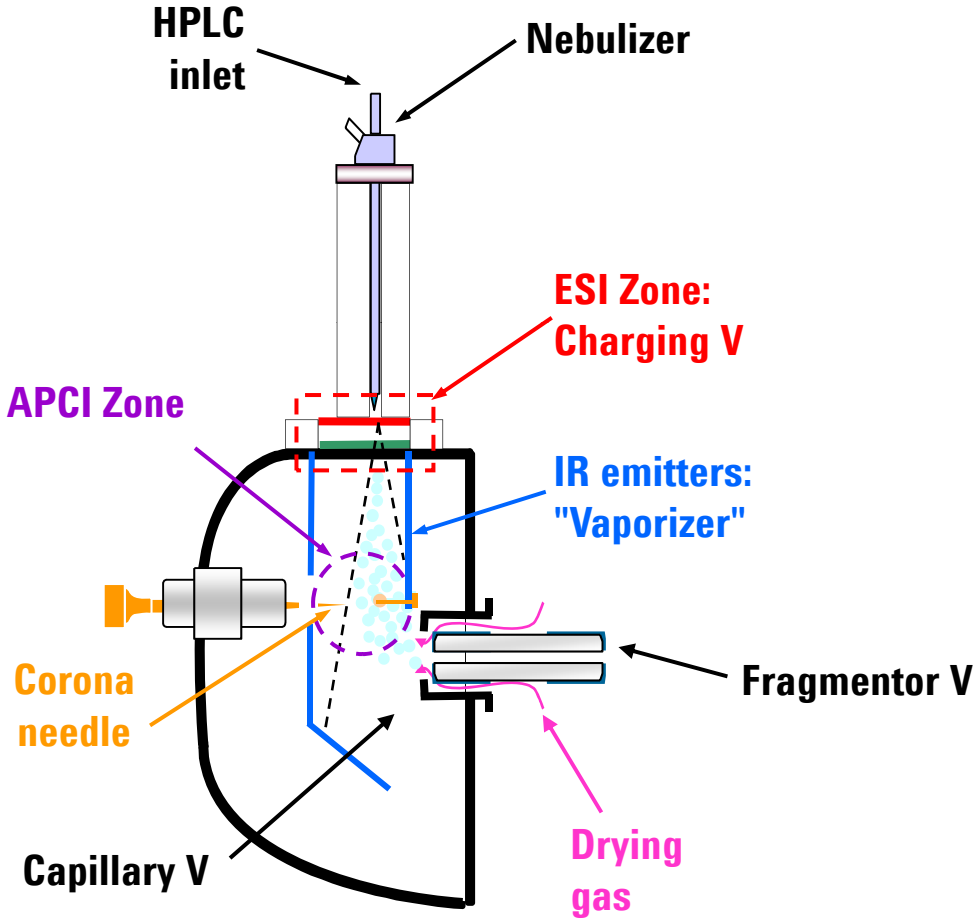
- QQQ method development and optimization
  - Source optimization for standard and Agilent Jetstream sources
  - Compound optimization using flow injection or Optimizer software
- MassHunter Quant software
  - Current revision B.03.02: What's New
  - Review of Quant methods: creation from acquired data, existing batch
  - Global and Advanced parameters
  - Optimizing peak identification and rejection
  - Quant method troubleshooting: identification and integration

Agilent ESI and APCI sources: for polar to non-polar compounds



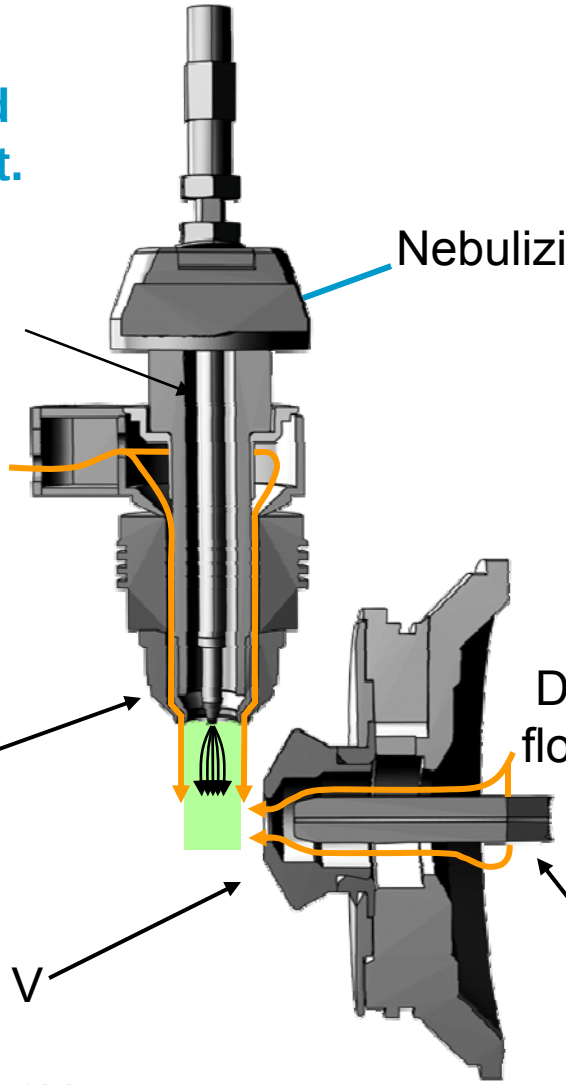


Agilent Multimode source for flexibility
ESI or APCI or Mixed mode (simultaneous ESI/APCI)



Agilent Jetstream Technology (AJT)

The super-heated sheath gas collimates the nebulizer spray and presents more ions to the MS inlet.



Enhanced efficiency nebulizer

Nebulizing gas: pressure

*Sheath gas: flow and temperature

*Nozzle voltage

Drying gas: flow and temperature

Resistive sampling capillary entrance: Capillary V

Resistive sampling capillary exit: FragV

*New parameters unique to AJT source

Source optimization for Agilent LC/MS systems
ESI, APCI, MM and Agilent Jetstream Technology (AJT)

Flow dependent parameters:

- Nebulizer pressure
- Drying gas temperature and flow
- Vaporizer temperature (MM)
- Sheath gas flow (AJT)

Compound dependent parameters:

- Capillary voltage
- Fragmentor voltage
- Collision Energy (QQQ, QTOF)
- Vaporizer temp (APCI, MM, AJT)
- Sheath gas temperature (AJT)
- Nozzle voltage (AJT)

Starting parameters for Agilent sources (small molecules)

Parameter	ESI source	APCI source
Nebulizer pressure	25 psi 0.2 mL/min 50 psi 1 mL/min	60 psi
Drying gas flow	10 Lpm ≤ 0.2 mL/min 12 Lpm > 0.4 mL/min	5 Lpm
Drying gas temp	325-350°C	350°C
Capillary voltage	3500V	3500V
Fragmentor voltage compound dependent	70-100 V Single quads 120-140V QQQ, TOF 140-170V 6230, 6530	70-100 V Single quads 120-140V QQQ, TOF 140-170V 6230, 6530

Starting parameters for Agilent sources (small molecules)

Parameter	Multimode source	Agilent Jetstream
Nebulizer pressure	60 psi ESI 20 psi APCI 40 psi Mixed mode	45 psi
Drying gas flow	5 Lpm	7 Lpm
Drying gas temp	250°C ESI, Mixed 300°C APCI	300°C
Capillary voltage	2000V	3500V
Charging voltage (MM)	2000V	
Nozzle voltage (AJT)		500V
Fragmentor voltage Compound dependent	70-100 V Single quads 120-140V QQQ, TOF 140-170V 6230, 6530	70-100 V Single quads 120-140V QQQ, TOF 140-170V 6230, 6530

Tuning and Calibration for Agilent QQQ systems

•Agilent QQQ systems are very stable and require infrequent Autotuning (if not, place a service call!).

•Current software uses "ESI-Low" tune mix for all QQQs.

•Routine use only requires resolution and mass axis verification for polarity and resolution modes to be used.

- Run performance check sample (lab SOP)
- Do Checktune (checks all three resolution modes)
- In Manual Tune, turn on calibrant and observe MS1 and MS2 profiles. Use *Adjust Gain and Offset* button if width or mass axis requires adjustment.

•If using negative ion, Autotuning should be done monthly or less to minimize source exposure to TFA.

- Flush source extensively with LC flow after 30-minute Autotune before running samples in negative ion.

- Autotune at least quarterly to optimize ion transmission and update EM voltage.

Optimizing the AJT source beginning with recommended starting values ( )

Order of effect on sensitivity	Things to note
Sheath gas temperature (250°C)	Requires time to stabilize
Sheath gas flow (8 Lpm)	Generally 10-12 Lpm for 0.2-0.7mL
Nebulizer pressure (45 psi)	LC flow- and mobile phase- dependent
Capillary voltage (3500V)	Somewhat MW dependent
Nozzle voltage (0V)	Very compound- and polarity-dependent
Drying gas temperature (250°C)	Interaction with flow and sheath parms
Drying gas flow (5 Lpm)	Generally 5-7 Lpm, higher when in doubt

Optimizing the AJT source
Typical values ( ) and Increments

Order of effect on sensitivity	Increments and ranges to test
Sheath gas temperature (325°C)	50°C, 250-400°C
Sheath gas flow (10 Lpm)	2 Lpm, 8 -12 Lpm
Nebulizer pressure (40 psi)	5 psi, 25 – 50 psi
Capillary voltage (3500V)	500V, 2500-4500V
Nozzle voltage (0V)	500V, 0 - 2000V
Drying gas temperature (300°C)	50°C, 250-350°C
Drying gas flow (7 Lpm)	2 Lpm, 5-11 Lpm

Optimization techniques

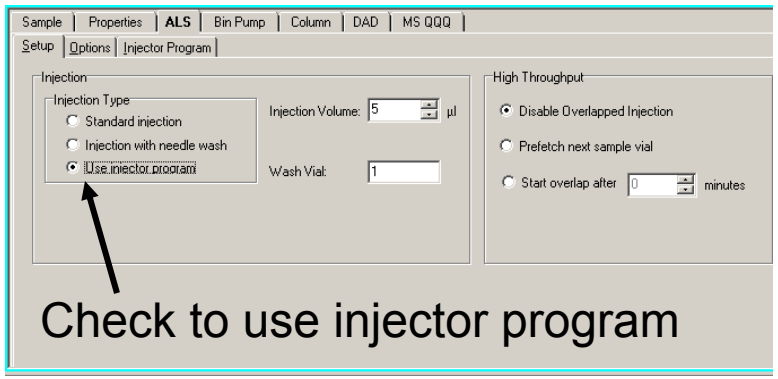
•Fragmentor V and Collision Energy (for MS/MS systems) should be optimized first using recommended starting parameters for source.

•Voltages, gas flows change quickly and can be optimized with series of flow injections using method with injector program and time segments.

•Temperatures (sheath gas, drying gas, vaporizers) require equilibration time between injections; perhaps best done with final chromatography conditions.

• Template flow injection methods also come with Optimizer software

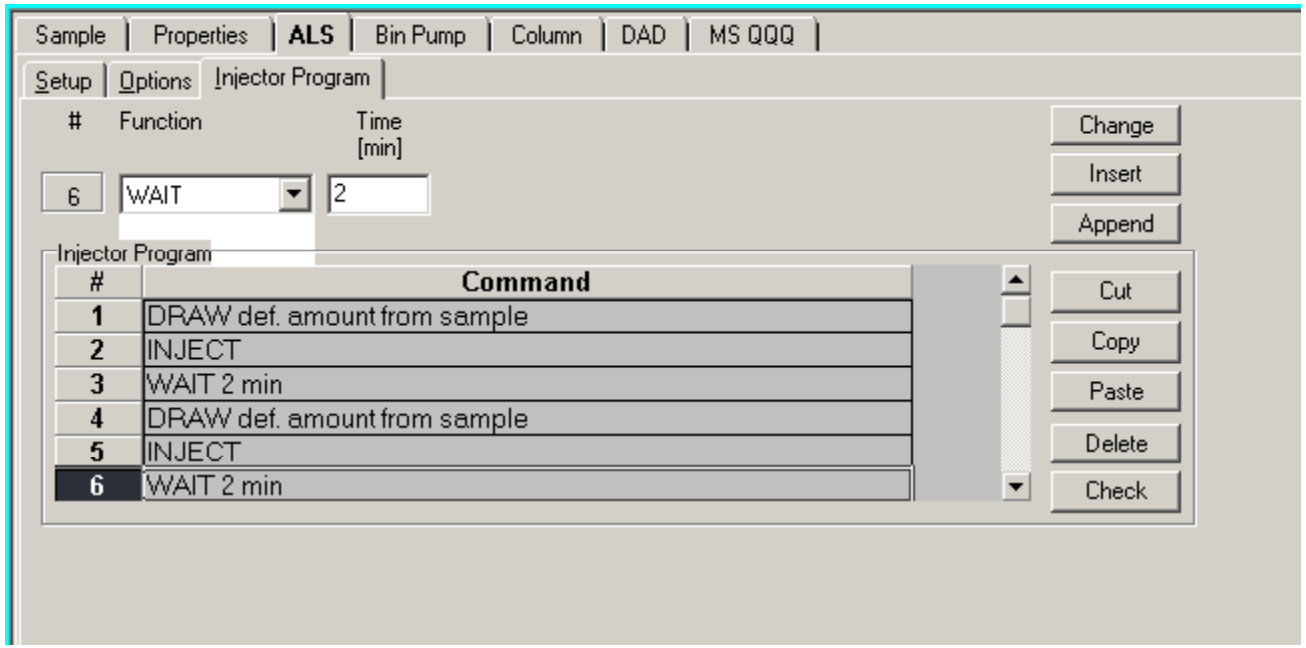
Injector program for series of flow injections



Injector program steps:

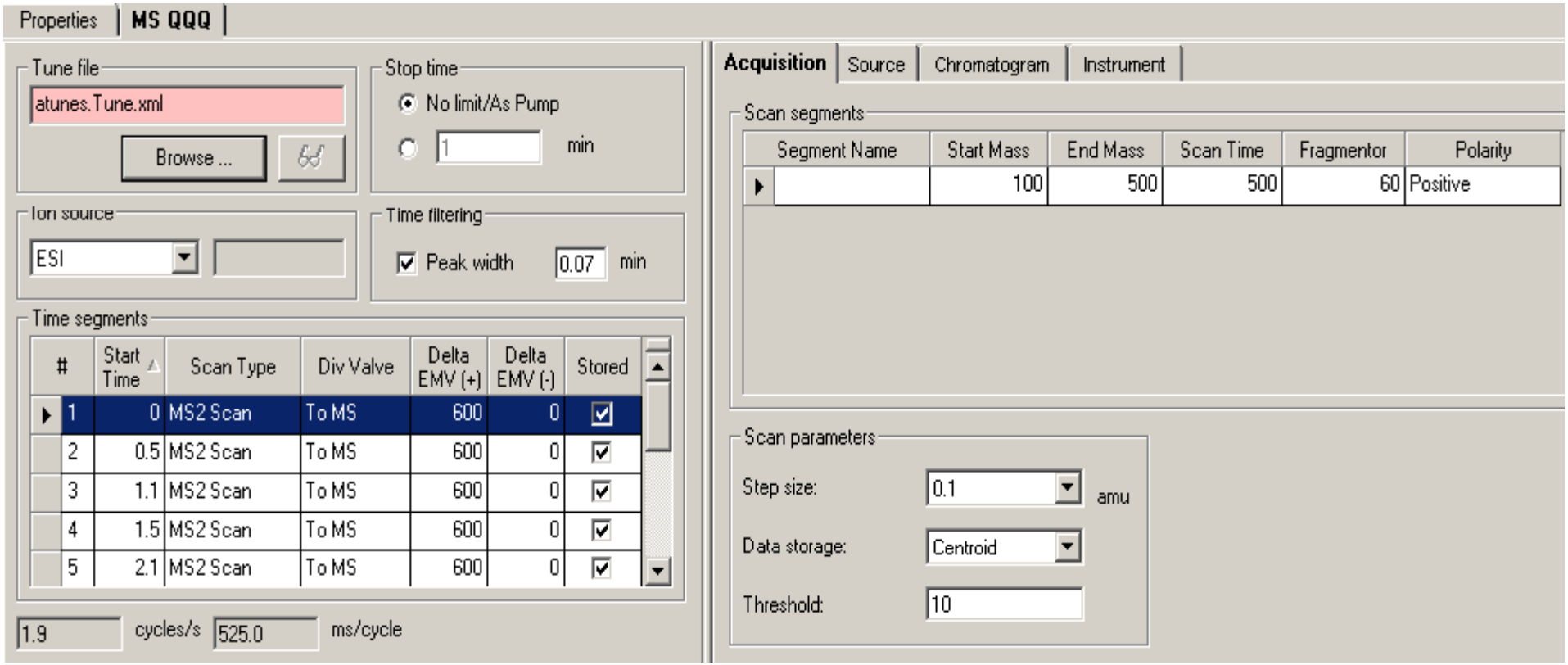
Valve to Bypass
Remote Startpulse
Repeat n times [n = number of time segments]
Draw default amount from sample
Valve to Mainpass
Wait 0.25 min [adjust to sync with acq time segments]
End repeat

Example Injector Program with 2-min spacing and explicit repeats



Note: *Inject* and *Remote Startpulse* commands work differently on QQQ and TOF/QTOF.

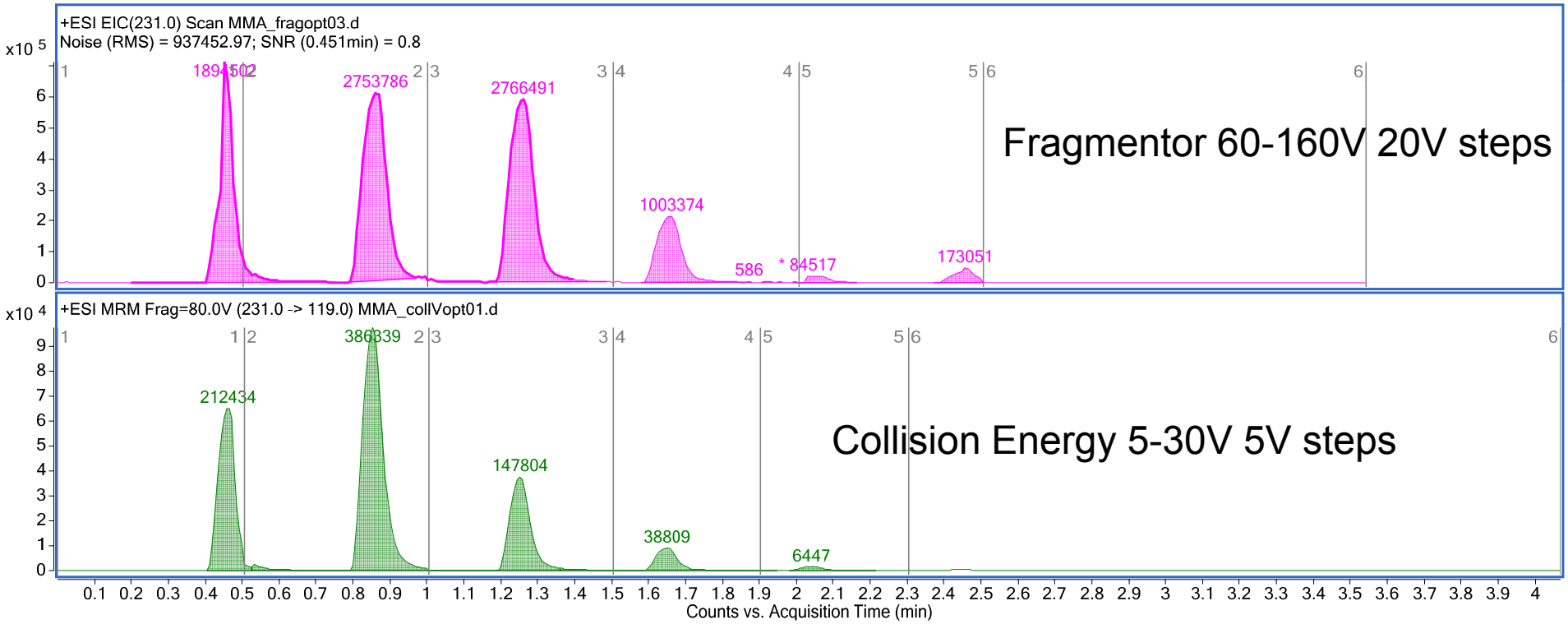
Injector program for series of flow injections Synchronize with MS time segments



Fragmentor and Collision Energy optimization using flow injection with injector program

Methylmalonic acid, dibutyl ester

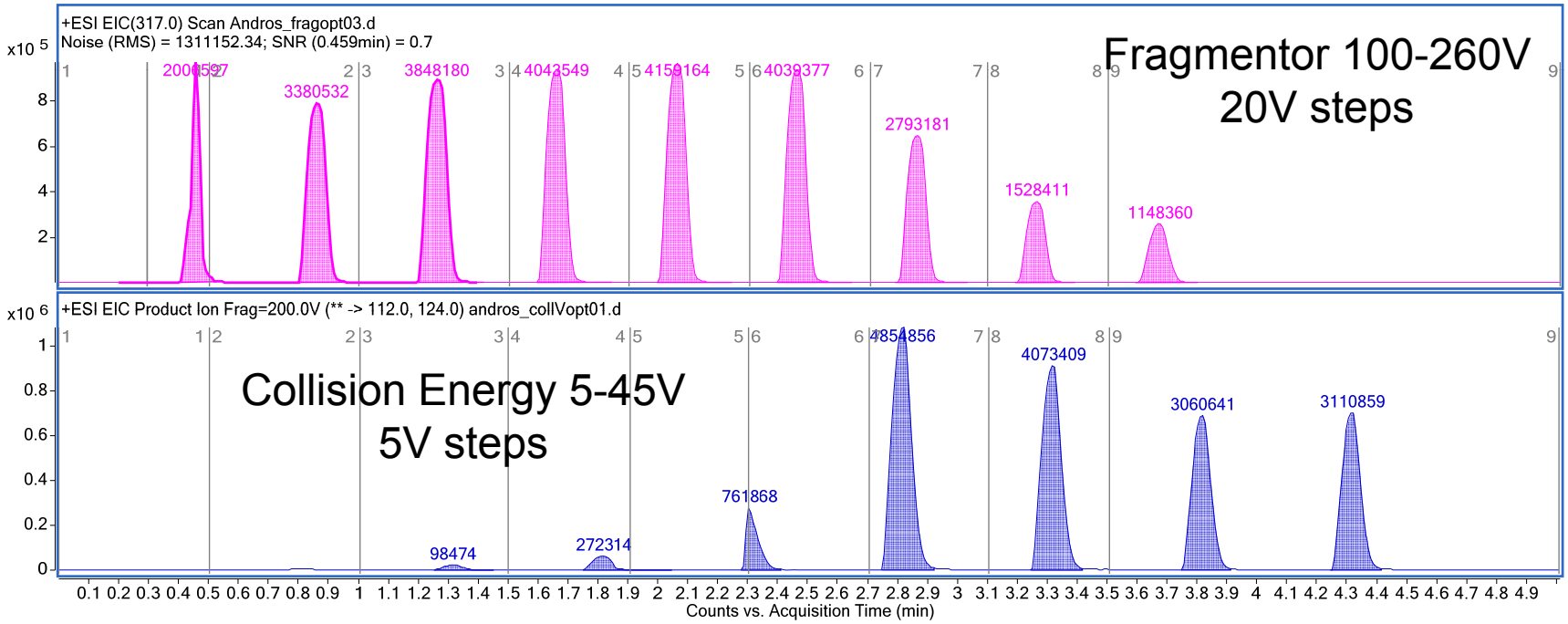
Maximize MH+ ion transmission, minimize CID with Fragmentor voltage
Maximize product ion signal(s) with Collision Energy
Can fine tune each with smaller steps after initial experiments



Fragmentor and Collision Energy optimization using flow injection with injector program

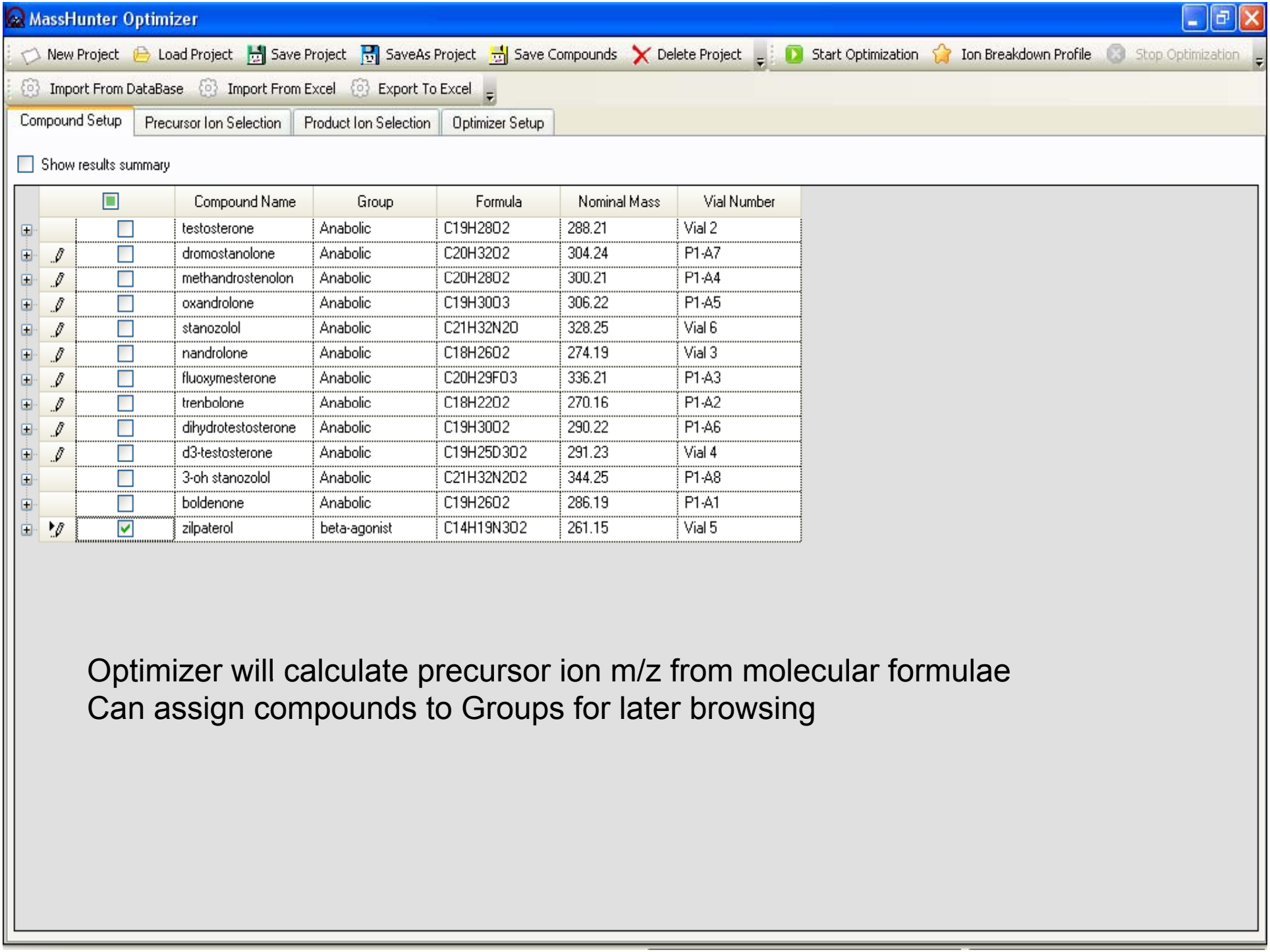
Androstenedione

Maximize MH+ ion transmission, minimize CID with Fragmentor voltage
Maximize product ion signal(s) with Collision Energy
Can fine tune each with smaller steps after initial experiments



Fragmentor and Collision Energy Determination with Optimizer software

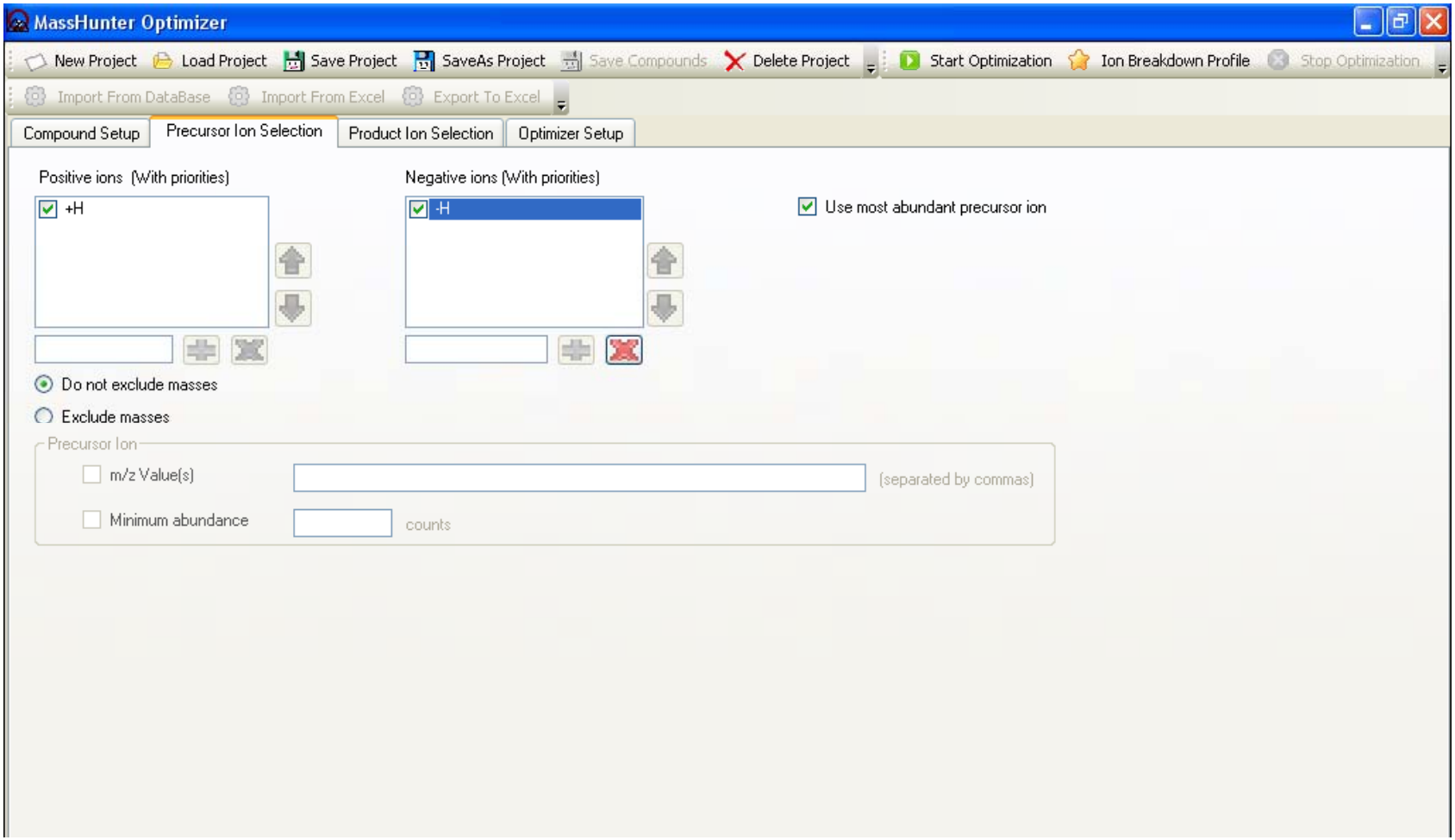
- Optional add-on to MassHunter QQQ acquisition software
- Current version: B.02.01 (requires B.02.01 Acquisition)
- Useful for frequent method development [new compound each week] or for multi-compound methods [e.g. toxicology, environmental applications]
- Many toxicology and pesticide transitions are available in new Agilent application kits
- Can use Flow Injection before LC separation is developed
- Can use LC method to optimize multiple compounds in a few injections


MassHunter Optimizer
New Project
Load Project
Save Project
SaveAs Project
Save Compounds
Delete Project
Start Optimization
Ion Breakdown Profile
Stop Optimization
Import From DataBase
Import From Excel
Export To Excel
Compound Setup
Precursor Ion Selection
Product Ion Selection
Optimizer Setup
Show results summary
Compound Name
Group
Formula
Nominal Mass
Vial Number
testosterone Anabolic C19H28O2 288.21 Vial 2
dromostanolone Anabolic C20H32O2 304.24 P1-A7
methandrostenolon Anabolic C20H28O2 300.21 P1-A4
oxandrolone Anabolic C19H30O3 306.22 P1-A5
stanozolol Anabolic C21H32N2O 328.25 Vial 6
nandrolone Anabolic C18H26O2 274.19 Vial 3
fluoxymesterone Anabolic C20H29FO3 336.21 P1-A3
trenbolone Anabolic C18H22O2 270.16 P1-A2
dihydrotestosterone Anabolic C19H30O2 290.22 P1-A6
d3-testosterone Anabolic C19H25D3O2 291.23 Vial 4
3-oh stanozolol Anabolic C21H32N2O2 344.25 P1-A8
boldenone Anabolic C19H26O2 286.19 P1-A1
zilpaterol beta-agonist C14H19N3O2 261.15 Vial 5
Optimizer will calculate precursor ion m/z from molecular formulae
Can assign compounds to Groups for later browsing

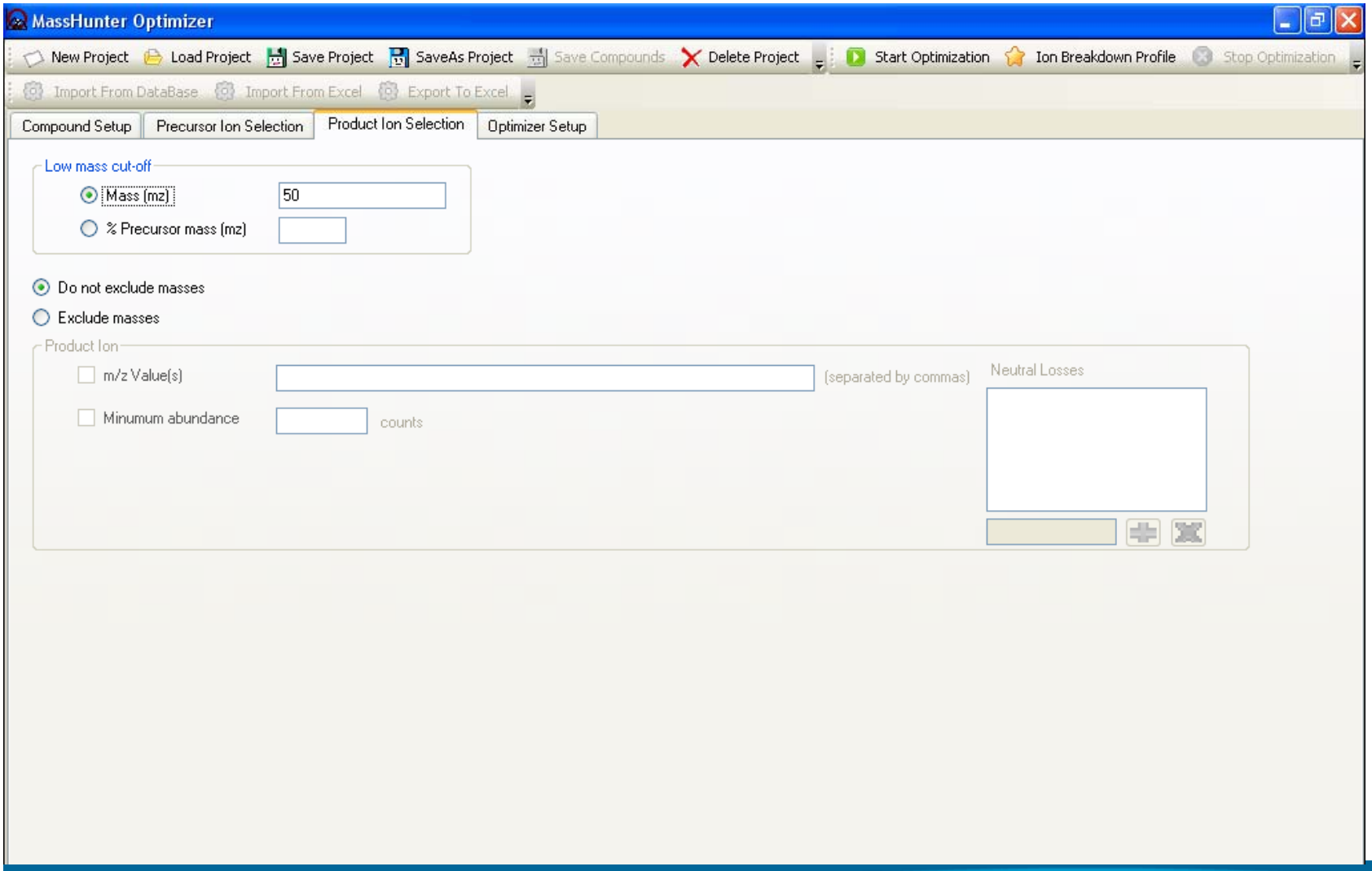
MassHunter Optimizer – precursor ion selection

User-selectable adducts (H, Na, K, OAc^- , $HCOO^-$, etc.)

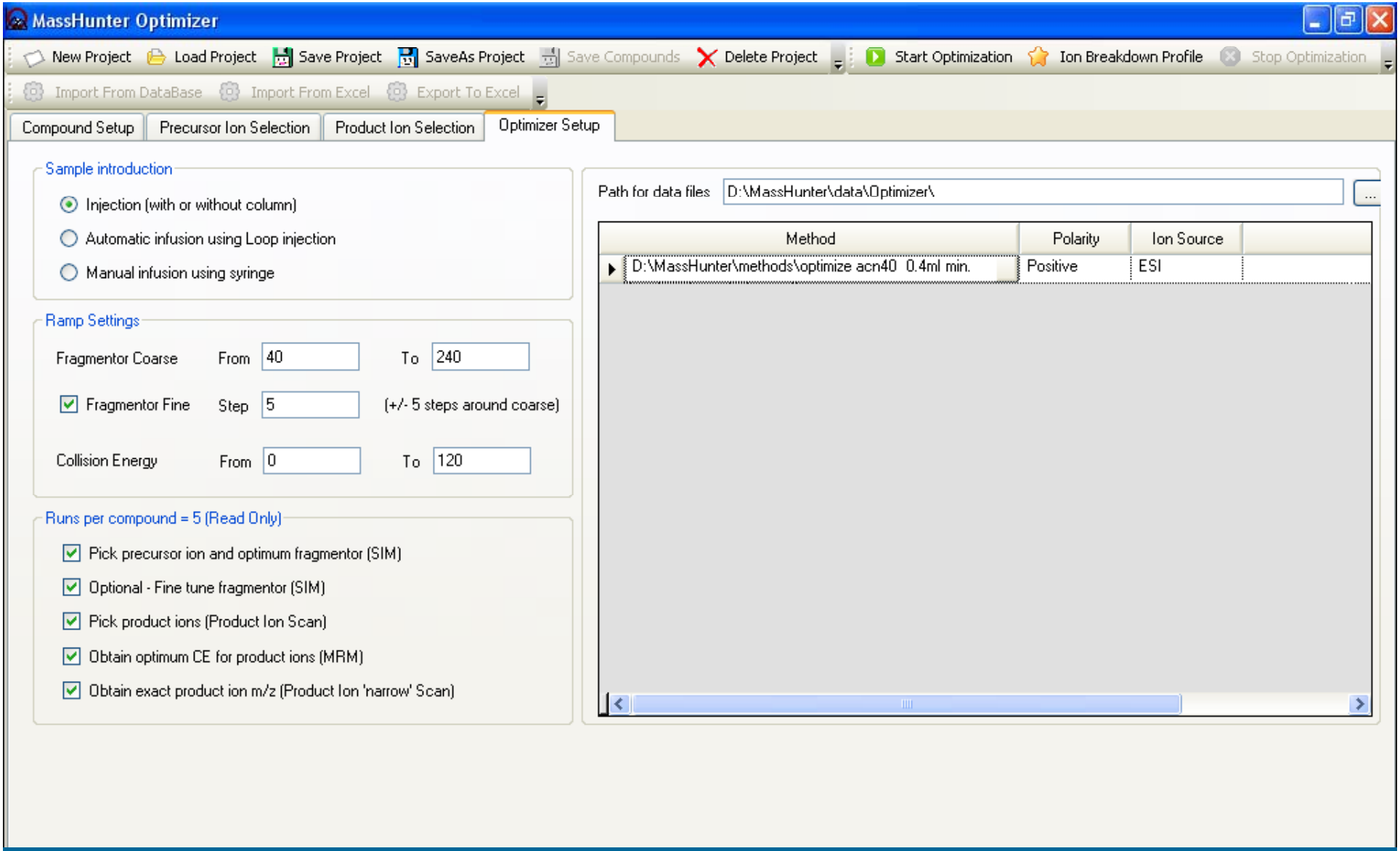
Can run both positive and negative ion methods in same Project



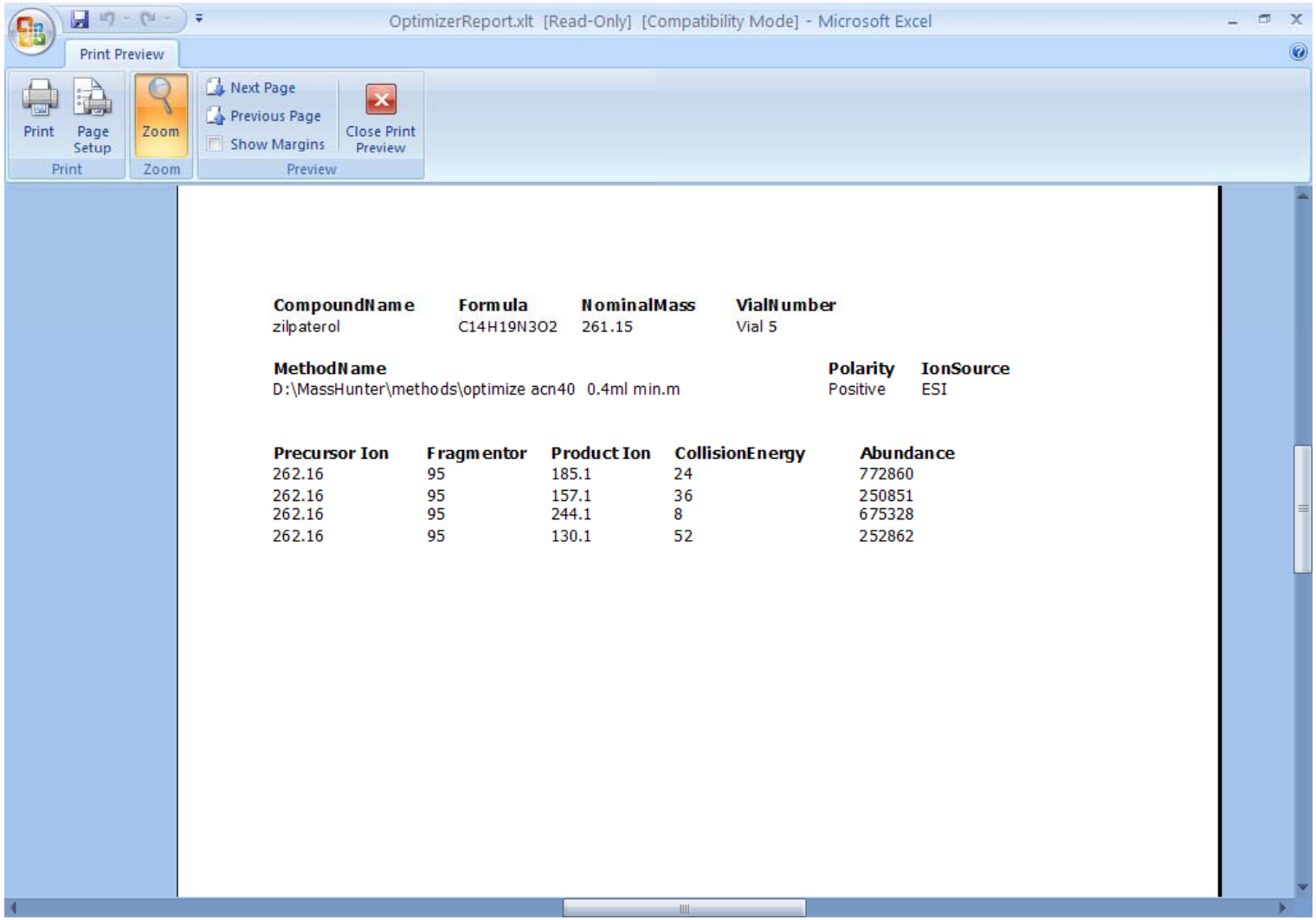
Optimizer – product ion selection and rejection (set mass range, minimum abundance)



Optimizer – method type, parameter step selection



Optimizer results
Automatically added to MRM database

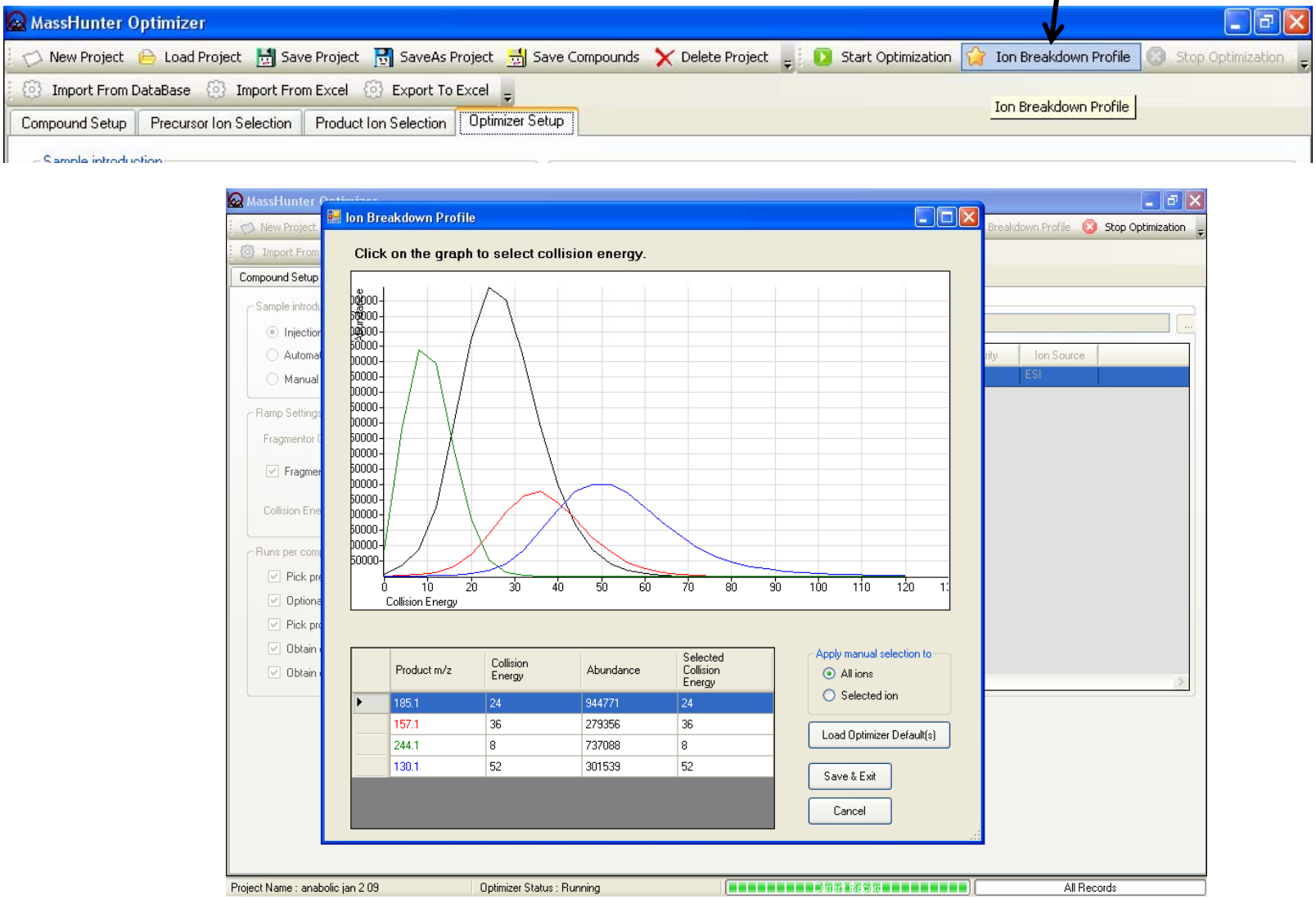


CompoundName	Formula	NominalMass	VialNumber
zilpaterol	C14H19N3O2	261.15	Vial 5

MethodName	Polarity	IonSource
D:\MassHunter\methods\optimize acn40 0.4ml min.m	Positive	ESI

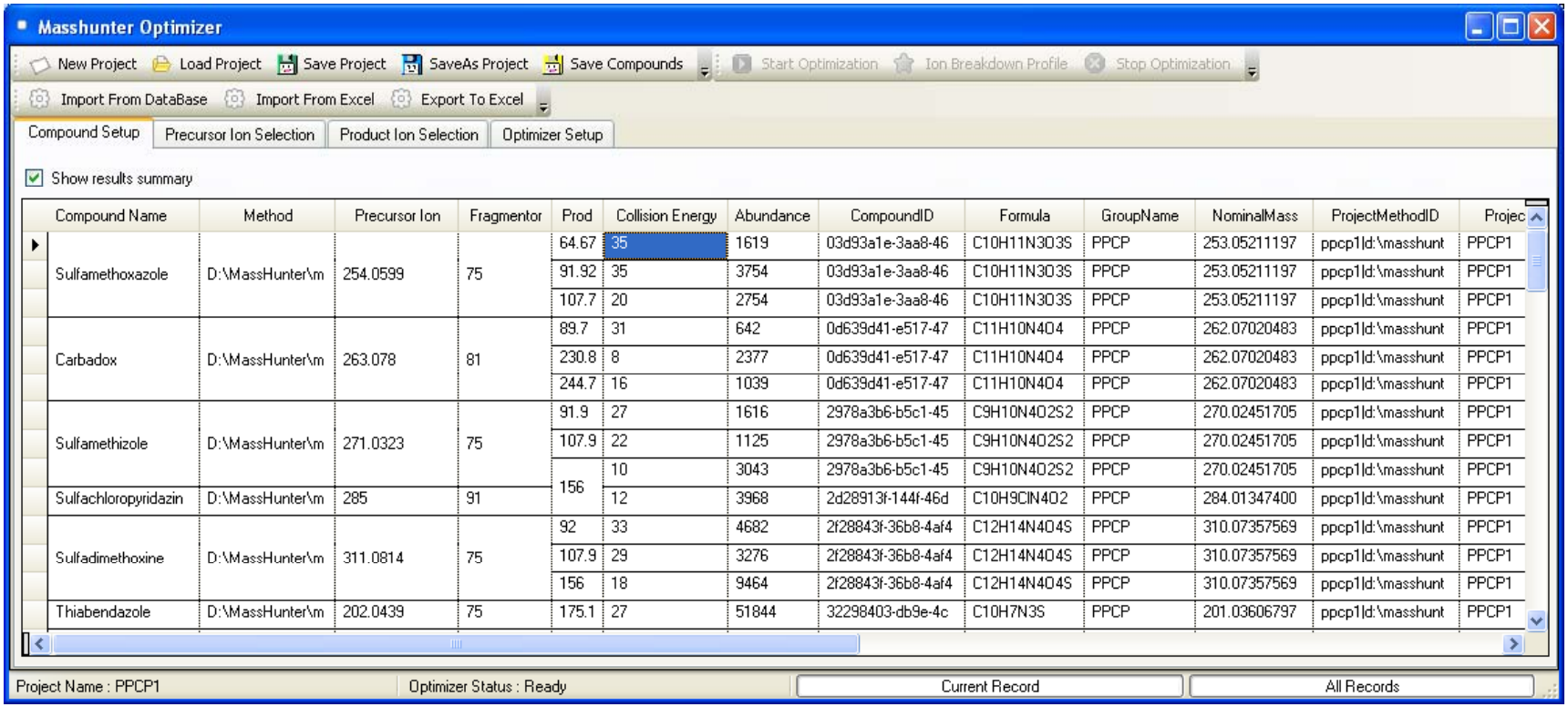
Precursor Ion	Fragmentor	Product Ion	CollisionEnergy	Abundance
262.16	95	185.1	24	772860
262.16	95	157.1	36	250851
262.16	95	244.1	8	675328
262.16	95	130.1	52	252862

Optimizer – interactive parameter selection



MassHunter Optimizer

Results for the project can be viewed immediately after run: precursor and product ions, Fragmentor and Collision Energies



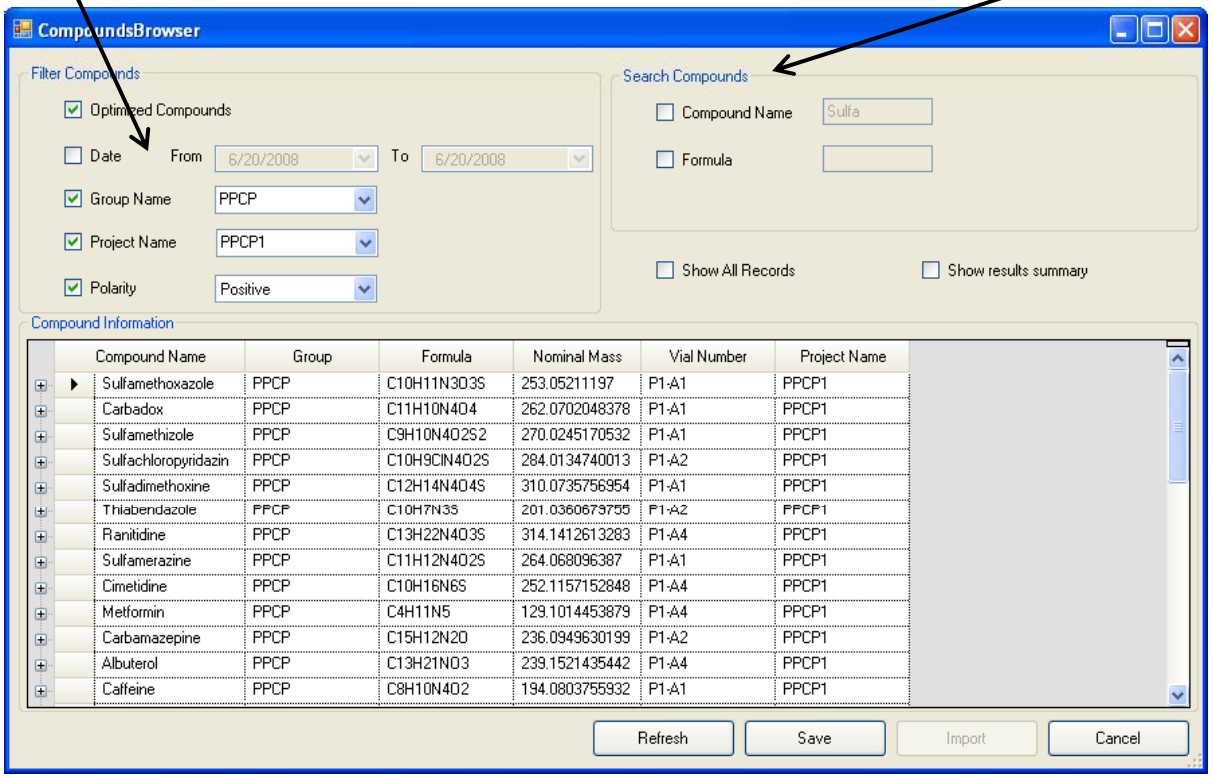
Compound Name	Method	Precursor Ion	Fragmentor	Prod	Collision Energy	Abundance	CompoundID	Formula	GroupName	NominalMass	ProjectMethodID	Projec
Sulfamethoxazole	D:\MassHunter\m	254.0599	75	64.67	35	1619	03d93a1e-3aa8-46	C10H11N3O3S	PPCP	253.05211197	ppcp1Id:\masshunt	PPCP1
				91.92	35	3754	03d93a1e-3aa8-46	C10H11N3O3S	PPCP	253.05211197	ppcp1Id:\masshunt	PPCP1
				107.7	20	2754	03d93a1e-3aa8-46	C10H11N3O3S	PPCP	253.05211197	ppcp1Id:\masshunt	PPCP1
Carbadox	D:\MassHunter\m	263.078	81	89.7	31	642	0d639d41-e517-47	C11H10N4O4	PPCP	262.07020483	ppcp1Id:\masshunt	PPCP1
				230.8	8	2377	0d639d41-e517-47	C11H10N4O4	PPCP	262.07020483	ppcp1Id:\masshunt	PPCP1
				244.7	16	1039	0d639d41-e517-47	C11H10N4O4	PPCP	262.07020483	ppcp1Id:\masshunt	PPCP1
Sulfamethizole	D:\MassHunter\m	271.0323	75	91.9	27	1616	2978a3b6-b5c1-45	C9H10N4O2S2	PPCP	270.02451705	ppcp1Id:\masshunt	PPCP1
				107.9	22	1125	2978a3b6-b5c1-45	C9H10N4O2S2	PPCP	270.02451705	ppcp1Id:\masshunt	PPCP1
				156	10	3043	2978a3b6-b5c1-45	C9H10N4O2S2	PPCP	270.02451705	ppcp1Id:\masshunt	PPCP1
Sulfachloropyridazin	D:\MassHunter\m	285	91		12	3968	2d28913f-144f-46d	C10H9ClN4O2	PPCP	284.01347400	ppcp1Id:\masshunt	PPCP1
Sulfadimethoxine	D:\MassHunter\m	311.0814	75	92	33	4682	2f28843f-36b8-4af4	C12H14N4O4S	PPCP	310.07357569	ppcp1Id:\masshunt	PPCP1
				107.9	29	3276	2f28843f-36b8-4af4	C12H14N4O4S	PPCP	310.07357569	ppcp1Id:\masshunt	PPCP1
				156	18	9464	2f28843f-36b8-4af4	C12H14N4O4S	PPCP	310.07357569	ppcp1Id:\masshunt	PPCP1
Thiabendazole	D:\MassHunter\m	202.0439	75	175.1	27	51844	32298403-db9e-4c	C10H7N3S	PPCP	201.03606797	ppcp1Id:\masshunt	PPCP1

Optimizer

Browse compounds in Acquisition for import

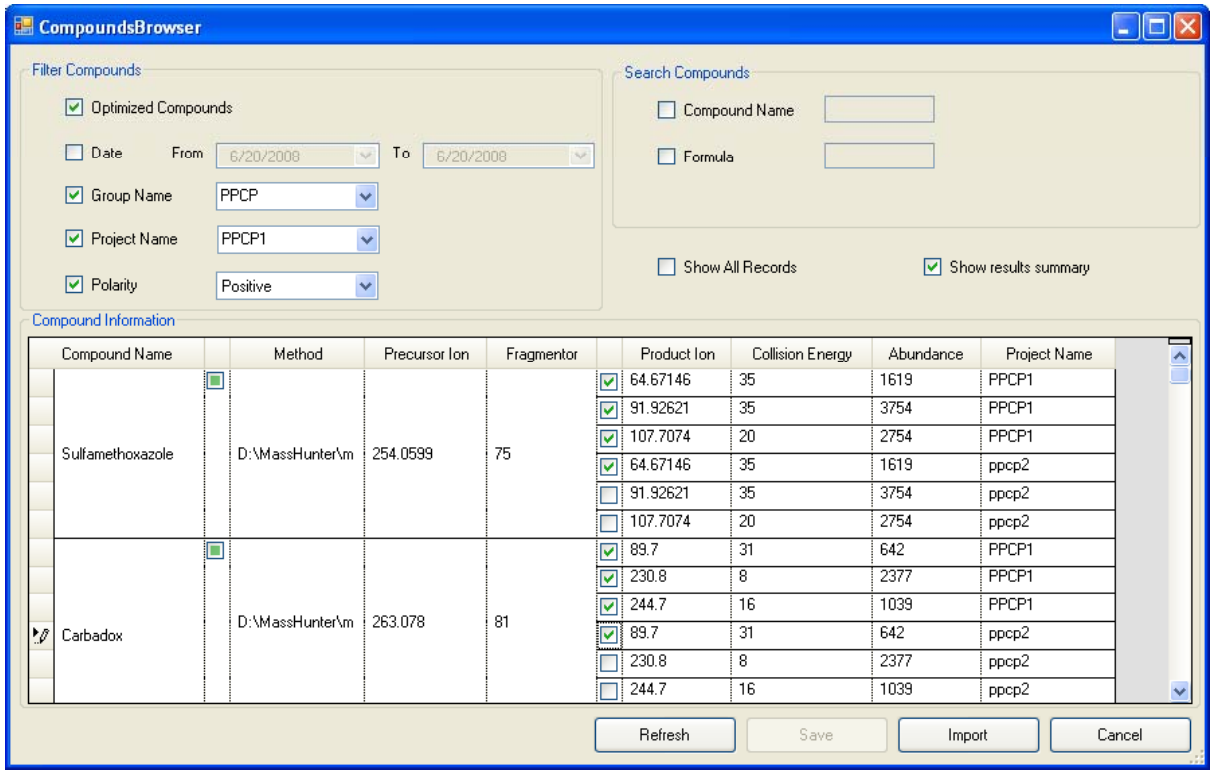
Compounds can be filtered by project, group, polarity or date

Compounds can be searched for by name or formula



MassHunter Optimizer

Build LC/MS/MS method from compound database



- Select compounds in database after filtering
- Select transitions for each compound (all or individual)
- Easily import into template LC/MS method

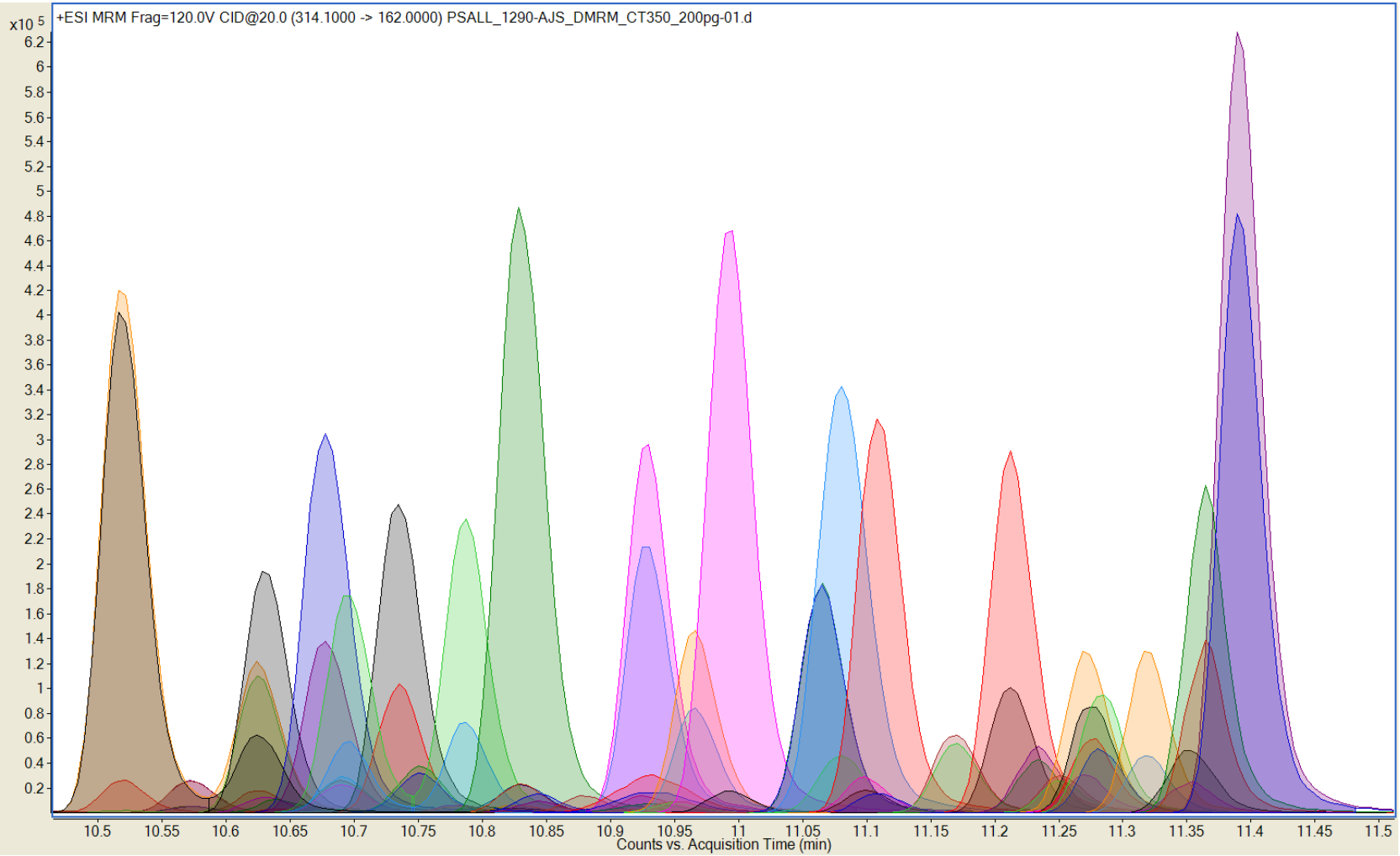
Dynamic MRM

Optimization of MRM Acquisition

- Good quantitation requires adequate number of data points across each peak (ideally 10-20)
- High confidence or regulated identification requires > 1 MRM per compound.
- Monitoring many compounds simultaneously lowers dwell time per MRM.
- Therefore for best sensitivity, only monitor compounds in retention time window where they elute.
- Traditional approach of time segments has limitations and is tedious to setup up and maintain.

Peak Capacities are very high with UHPLC

40 MRM Transitions in 1 minute time window



The solution: MassHunter Dynamic MRM

Included in QQQ Acquisition B.02.01

For applications requiring quantitation of 100 – 1000 compounds in one run; some examples:

- Food and environmental analysis (e.g. pesticides)
- Targeted quantitation of proteins via peptides (proteomics)

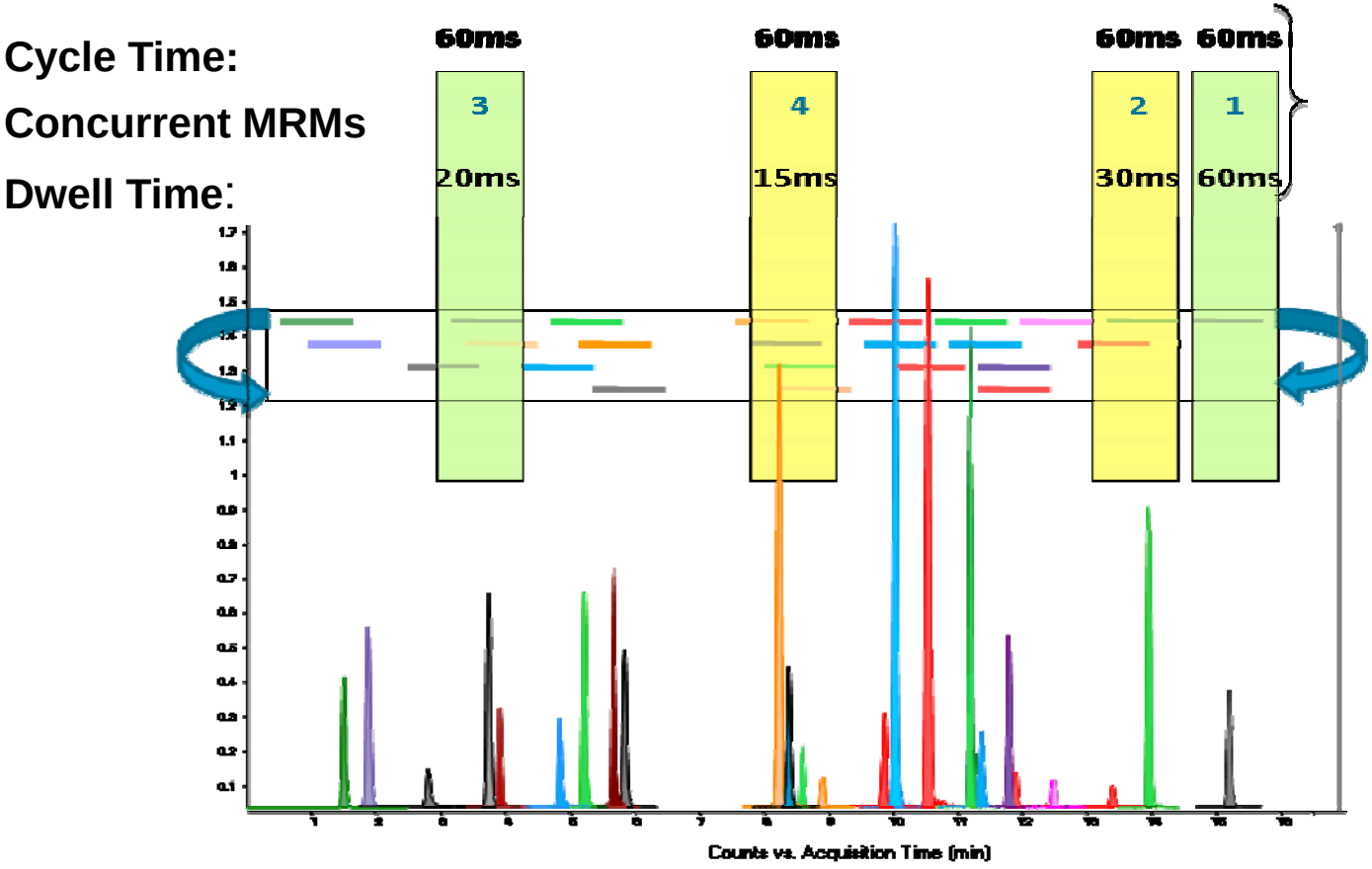
WITHOUT Dynamic MRM:

- Need to manually set up multiple time segments to maximize dwell times
- Tedious to set up; problematic if changes in retention times

WITH Dynamic MRM:

- Automatic setup of overlapping time segments without user intervention
- Fewer MRMs per unit time results in longer dwell time => incr sensitivity
- Unaffected by minor chromatographic time shifts

Dynamic MRM for 6400 Series Triple Quads
monitors transitions only when compounds elute



1. # Concurrent MRMs fewer than with time segments → more data points across each peak
2. Allows longer dwell times → better sensitivity, S/N

Acquisition setup with Dynamic MRM:

No time segments, instead

Retention Time and "Delta Ret Time" [MRM time range]

Acquisition | Source | Chromatogram | Instrument | Diagnostics

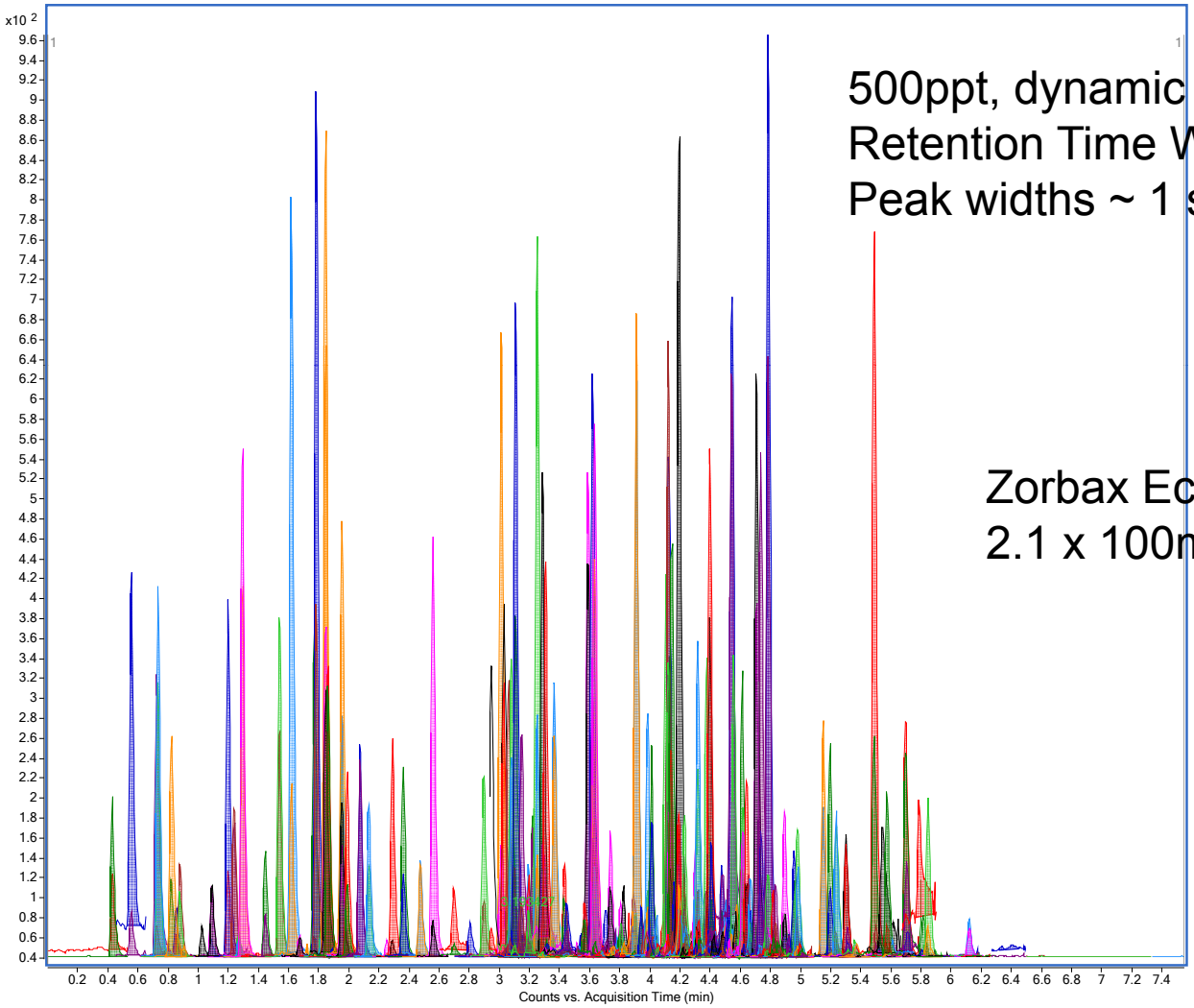
Scan segments

Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Fragmentor	Collision Energy	Ret Time (min)	Delta Ret Time	Polarity
Alprazolam	☐	309.1	Unit	281	Unit	179	25	3.715	1	Positive
Cocaine	☐	304.2	Unit	182.1	Unit	138	17	2.358	1	Positive
d-Amphetamine	☐	136.1	Unit	91	Unit	66	17	1.278	1	Positive
Diazepam	☐	285.1	Unit	154	Unit	169	25	4.269	1	Positive
Heroin	☐	370.2	Unit	165	Unit	149	61	2.236	1	Positive
Hydrocodone	☐	300.2	Unit	199	Unit	159	29	1.38	1	Positive
Lorazepam	☐	321	Unit	275	Unit	102	21	3.61	1	Positive
MDA	☐	180.1	Unit	163	Unit	61	5	1.311	1	Positive
MDEA	☐	208.1	Unit	163	Unit	107	9	1.72	1	Positive

Dynamic MRM Parameters

Cycle Time 500 ms

8 Minute Dynamic MRM Analysis with 6460 QQQ - 250-compound Pesticide Screen.



500ppt, dynamic MRM
Retention Time Window = 12 sec
Peak widths ~ 1 second

Zorbax Eclipse Plus C18
2.1 x 100mm (1.8µm)

Summary – Optimization of QQQ Acquisition

- Source optimization: flow and compound dependent parameters
- Agilent Jetstream Technology source: increased sensitivity vs regular ESI source if parameters optimized correctly:
  - Sheath gas temperature and flow are most important
  - Other parameters have less effect on response
  - Need less drying gas flow and temperature, but keep capillary clean
- Dynamic MRM is available for all 6400 models
  - More data points across peak
  - Longer dwell times for better sensitivity, S/N
  - Easier to set up and maintain than time segment methods

MassHunter Quantitative Software Review and Quant Method Optimization

Topics

- Method setup from acquired data
- Method re-use and updating
- Peak detection optimization
- Peak identification troubleshooting
- What's new in MH Quant B.03.02

Important MassHunter Quant concepts and rules: Batches

- A Batch is a file which contains all the Quant results from a set of data files AND the Quant method used. Very convenient for backup and moving data around.
- All the data files in a Batch must reside in a single directory, so put them all together before creating New Batch.
- Select the data directory BEFORE naming the new Batch
- Using the *Browse to Copy Samples* button when creating the Batch can be dangerous: you will have two copies of the same files (one with Quant results and one without)!

Important MassHunter Quant concepts and rules: Quant methods

- THE most common method problem: Calibration/QC level names in Batch and Quant method do not match, e.g.
  - Batch: levels are 1,2,…5, QC-Lo, QC-Hi
  - Method: levels are L1, L2…L5, QC-Low, QC-High
- 2nd most common problem: a hidden column in the Method with a key parameter, e.g. Criteria in MRM Compound Setup for peak selection, or Ion Polarity if method created manually.
- Many Quant parameters can be copied between compounds with *Apply to All* button.
- When in doubt, Right-Click to look for convenient features and shortcuts, like Fill Down or Fill Column.

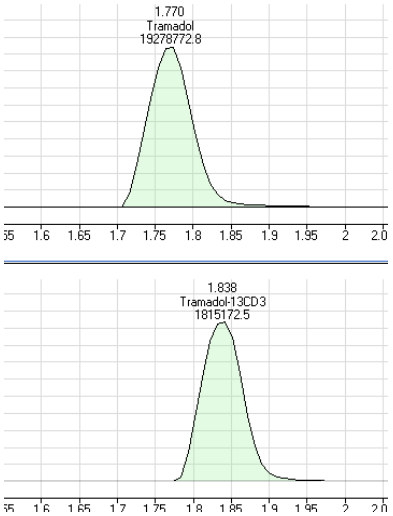
MassHunter Quant – optimizing target compound identification

Make the Criteria column visible in MRM Compound Setup:

Quantifier

Name	TS	Transition	Scan	Type	RT	Left RT Delta	Right RT Delta	RT Delta Units	Criteria
Tramadol-13CD3	2	268.2 -> 58.1	MRM	ISTD	1.850	0.500	0.500	Minutes	Greatest Response
Tramadol	2	264.2 -> 58.1	MRM	Target	1.850	0.500	0.500	Minutes	Greatest Response

Using the default of Greatest Response may result in the wrong peak being chosen as the target compound:



Endogenous interference with wrong retention time incorrectly identified as tramadol (no qualifier)

MassHunter Quant – optimizing target compound identification

Change the Criteria to be more specific:

Quantifier

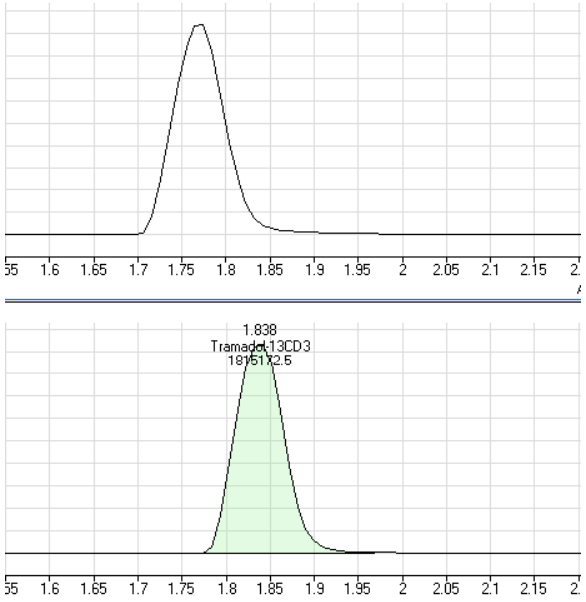
Name	TS	Transition	Scan	Type	RT	Left RT Delta	Right RT Delta	RT Delta Units	Criteria
Tramadol-13CD3	2	268.2 -> 58.1	MRM	ISTD	1.850	0.500	0.500	Minutes	Close RT
Tramadol	2	264.2 -> 58.1	MRM	Target	1.850	0.500	0.500	Minutes	Close RT

This might still not be selective enough, so specify how close the retention time must match the calibrated RT with Non-reference Window parameter in Globals:

Globals

Apply Multiplier to ISTD	☐
Apply Multiplier to Surrogate	☑
Apply Multiplier to Target	☑
Bracketing Type	None
Correlation Window	2.000
Ignore Peaks Not Found	☑
Non Reference Window	0.100
Non Reference Window Type	Minutes
Reference Window	0.100
Reference Window Type	Minutes
RelativeISTD	☐

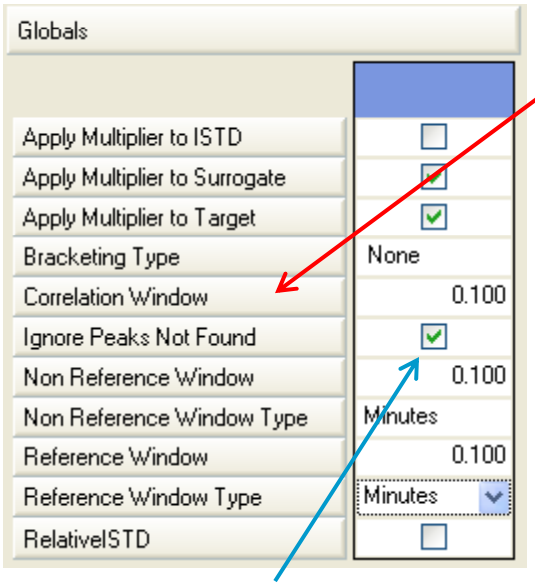
Now the offending peak with the wrong RT is ignored:



MassHunter Quant – optimizing target compound identification

Qualifying the Qualifier:

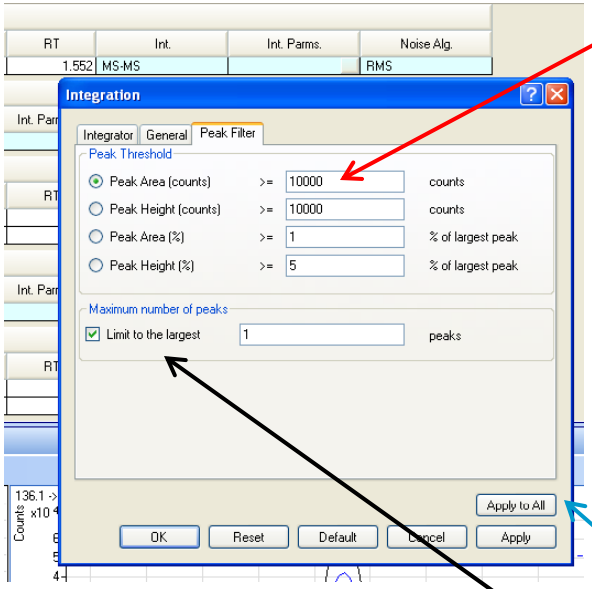
For qualifiers, you can ensure that the qualifier and the quant ion have the same retention time (i.e. line up properly as they should if coming from the same chromatographic peak), by setting the **Correlation Window** in Globals correctly. The default of 2 minutes is WAY too large.



The Ignore Peaks Not Found checkbox will prevent reporting "Amount=0.00" for target compounds not present with your criteria.

MassHunter Quant – Setting a reporting threshold:

You can also make Quant ignore peaks that meet these criteria but are below your desired detection or reporting threshold, or that really are background (if you can't use Qualifiers). Set a Peak Filter (area reject) value in *Advanced Tasks …Integration Parameters*.

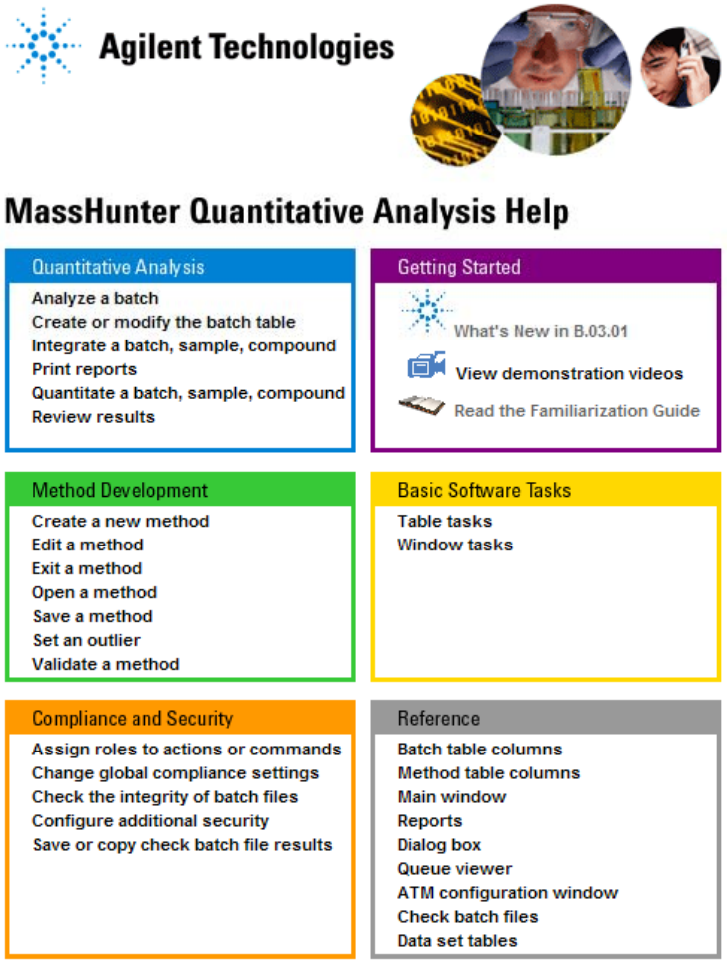


You can have the same Peak Filter for all compounds with Apply to All, or specific values for each compound. You can also limit integration to the N largest peak(s), like only one in this example.

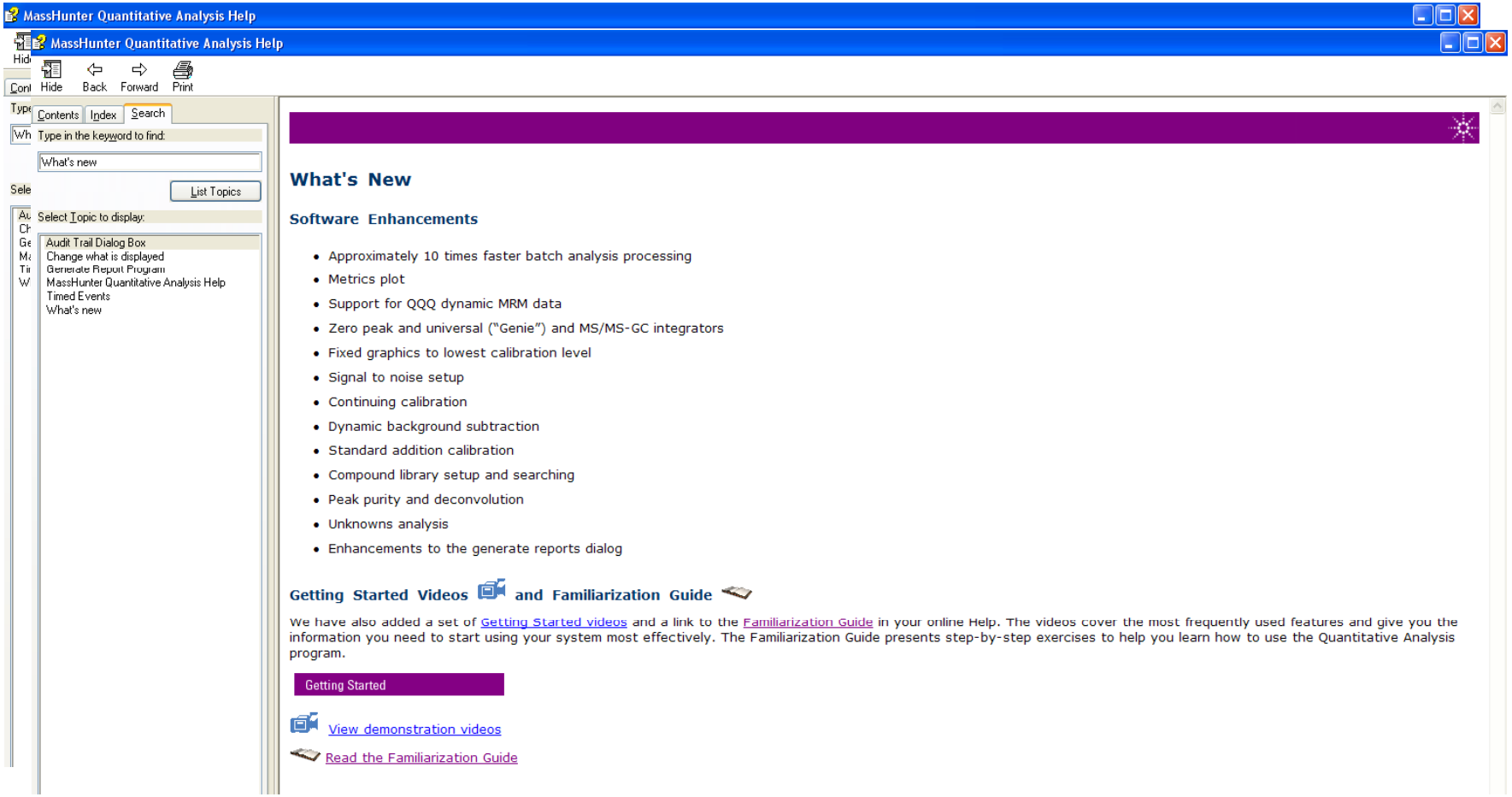
MassHunter Quantitation Integrators:

General
Universal
MS/MS – requires 64 data points within window

What's new in MassHunter Quant B.03.02 (just type 'What's new' in the Online Help!)



What's new in MassHunter Quant B.03.02
Something for everybody



What's new in MassHunter Quant B.03.02
More legibly:

Software Enhancements (some are instrument-specific)

- Approximately 10 times faster batch analysis processing
- Compound Math !
- Metrics plot (e.g. for IS areas)
- Support for QQQ dynamic MRM data
- More integrator choices: “Universal" (ChemStation) and GC/MS/MS integrators
- Fixed graphics to lowest calibration level
- Signal to noise setup
- Continuing calibration
- Dynamic background subtraction
- Standard addition calibration
- Compound library setup and searching
- Peak purity and deconvolution
- More choices in the Generate Reports dialog (e.g. no graphics)

- **Direct Link to *Getting Started Videos* and *Familiarization Guide***

Final recommendation for learning MassHunter Quant:

Uses data files included with the software