Quantification by LC-MS/MS for Pharma and Biopharma Applications

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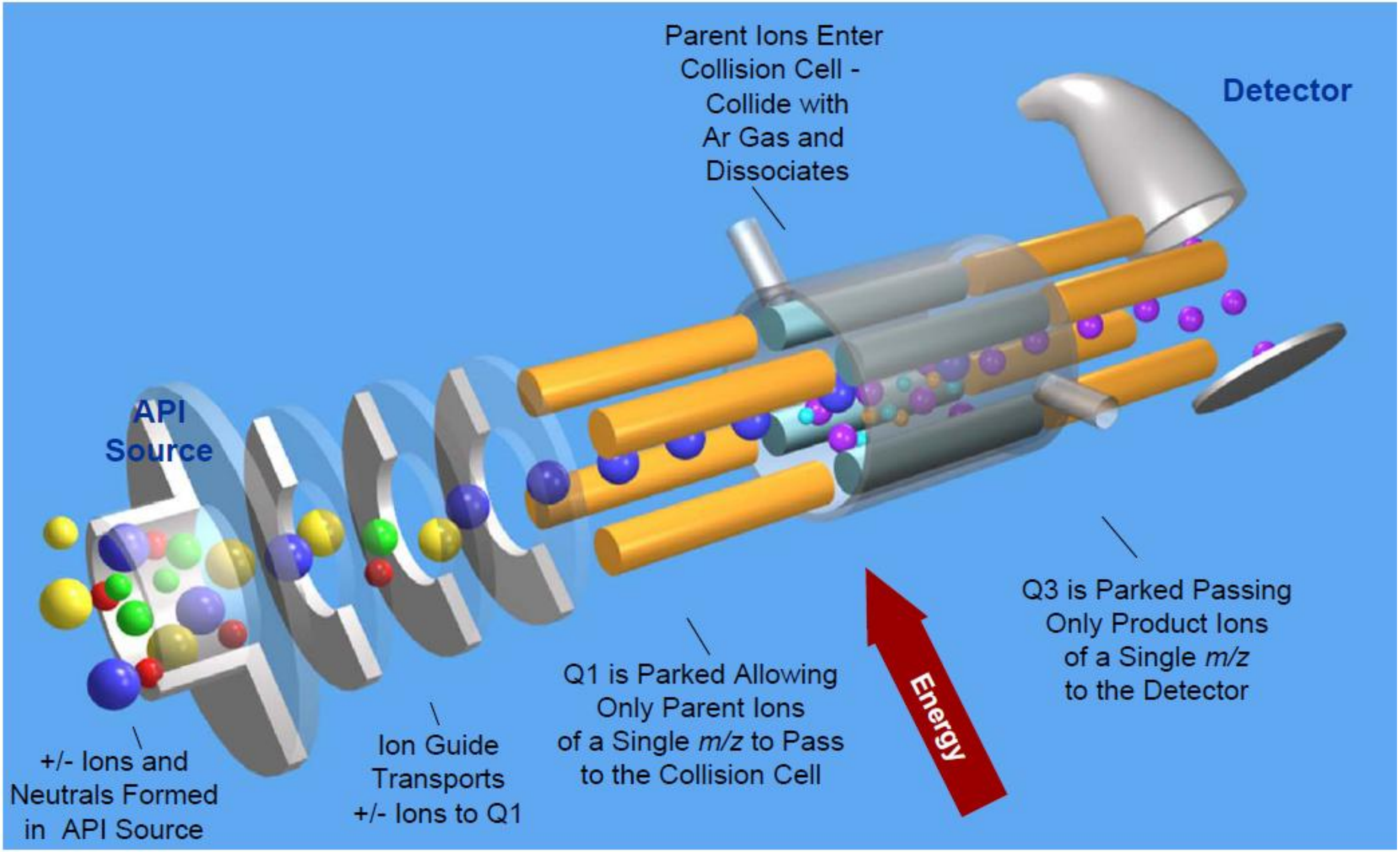
LC-MS Product Specialist CH

Overview

1. Introduction – QQQ for quantitation
2. Pharma applications
3. 1290 Infinity + 6490 QQQ application examples
4. Biopharma applications
5. HPLC-Chip + QQQ application examples

Triple Quadrupole: SRM



Parent Ions Enter
Collision Cell -
Collide with
Ar Gas and
Dissociates

Detector

API Source

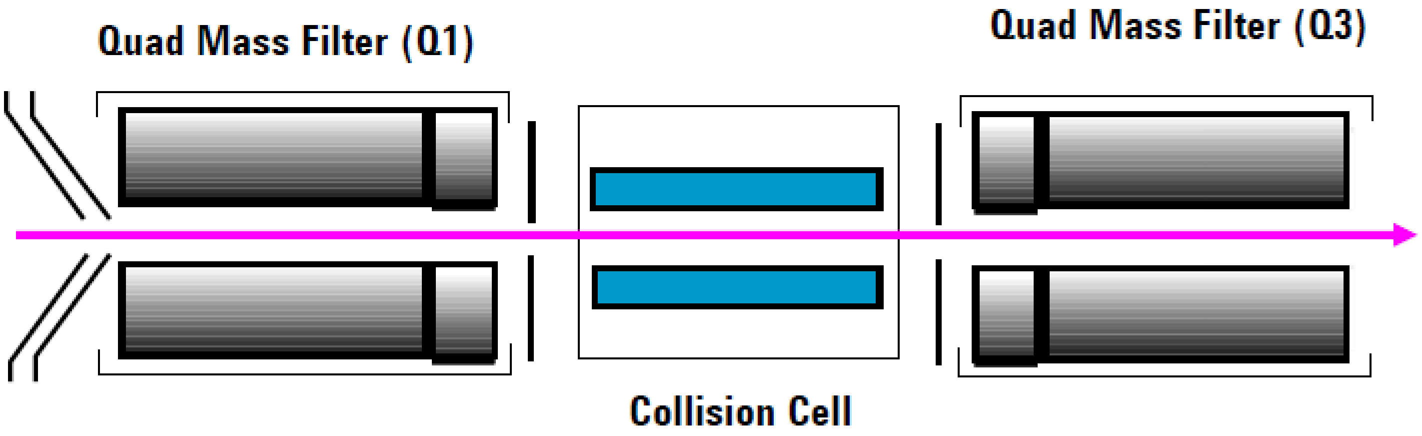
+/- Ions and
Neutrals Formed
in API Source

Ion Guide
Transports
+/- Ions to Q1

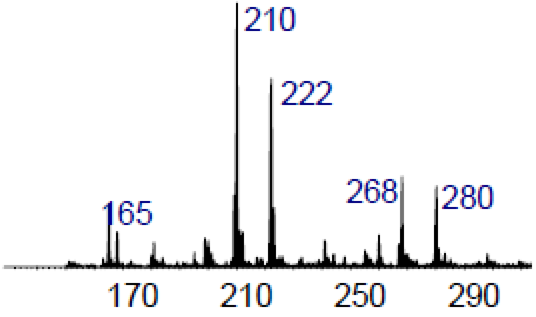
Q1 is Parked Allowing
Only Parent Ions
of a Single *m/z* to Pass
to the Collision Cell

Q3 is Parked Passing
Only Product Ions
of a Single *m/z*
to the Detector

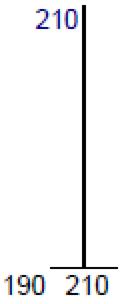
MRM (Multiple Reaction Monitoring)



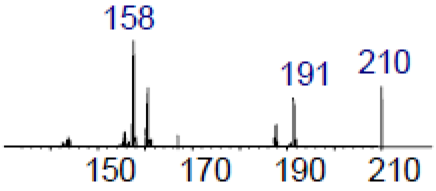
Spectrum with background ions (from ESI)



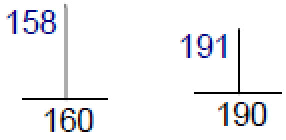
Q1 lets **only** target ion 210 pass through



Collision cell breaks ion 210 apart



Q3 monitors **only** characteristic fragments 158 and 191 from ion 210 for quant and qual.



no chemical background

Small molecules

PHARMA APPLICATIONS

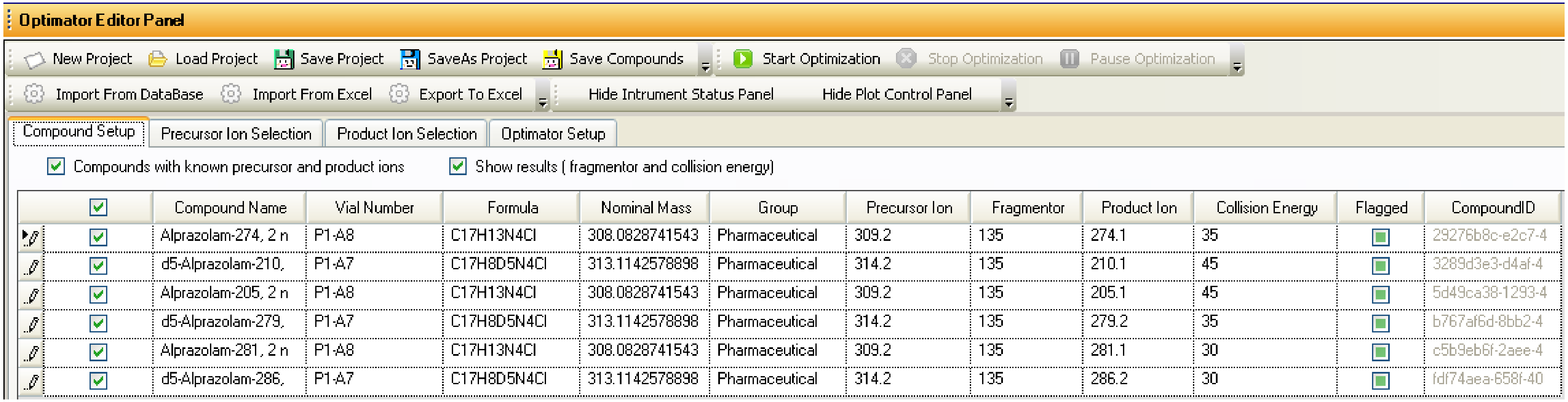
Main points on LC-QQQ

- Sensitivity
  - Extra sensitivity facilitates the sample preparation step
  - DMPK, ADME… LoD is critical

- Robustness
  - Source robustness: reproducibility over time
  - Required for method validation and long term studies

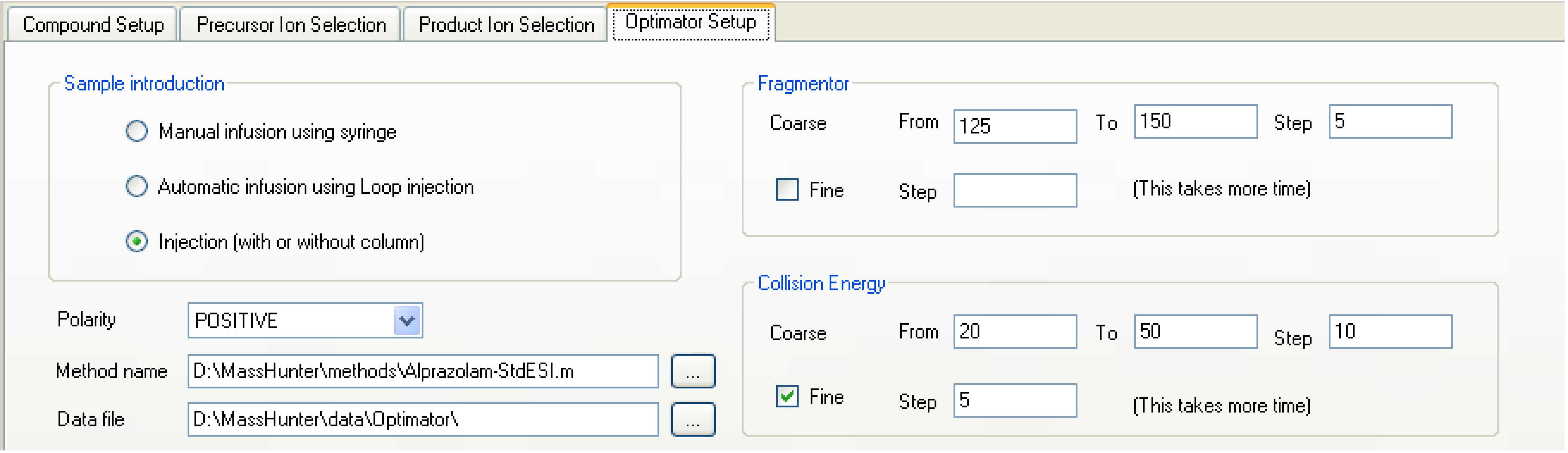
- Software
  - Ease of use – not everybody needs to be an expert
  - Data review in a quick and reliable manner
  - User friendly reporting – LIMS compatibility

Mass Hunter Optimizer
Compound Entry & Method Setup



☑ Compounds with known precursor and product ions ☑ Show results (fragmentor and collision energy)

☑	Compound Name	Vial Number	Formula	Nominal Mass	Group	Precursor Ion	Fragmentor	Product Ion	Collision Energy	Flagged	CompoundID
☑	Alprazolam-274, 2 n	P1-A8	C17H13N4Cl	308.0828741543	Pharmaceutical	309.2	135	274.1	35	🟩	29276b8c-e2c7-4
☑	d5-Alprazolam-210,	P1-A7	C17H8D5N4Cl	313.1142578898	Pharmaceutical	314.2	135	210.1	45	🟩	3289d3e3-d4af-4
☑	Alprazolam-205, 2 n	P1-A8	C17H13N4Cl	308.0828741543	Pharmaceutical	309.2	135	205.1	45	🟩	5d49ca38-1293-4
☑	d5-Alprazolam-279,	P1-A7	C17H8D5N4Cl	313.1142578898	Pharmaceutical	314.2	135	279.2	35	🟩	b767af6d-8bb2-4
☑	Alprazolam-281, 2 n	P1-A8	C17H13N4Cl	308.0828741543	Pharmaceutical	309.2	135	281.1	30	🟩	c5b9eb6f-2aee-4
☑	d5-Alprazolam-286,	P1-A7	C17H8D5N4Cl	313.1142578898	Pharmaceutical	314.2	135	286.2	30	🟩	fdf74aea-658f-40



Sample introduction

○ Manual infusion using syringe

○ Automatic infusion using Loop injection

● Injection (with or without column)

Polarity: POSITIVE

Method name: D:\MassHunter\methods\Alprazolam-StdESI.m

Data file: D:\MassHunter\data\Optimator\

Fragmentor

Coarse From 125 To 150 Step 5

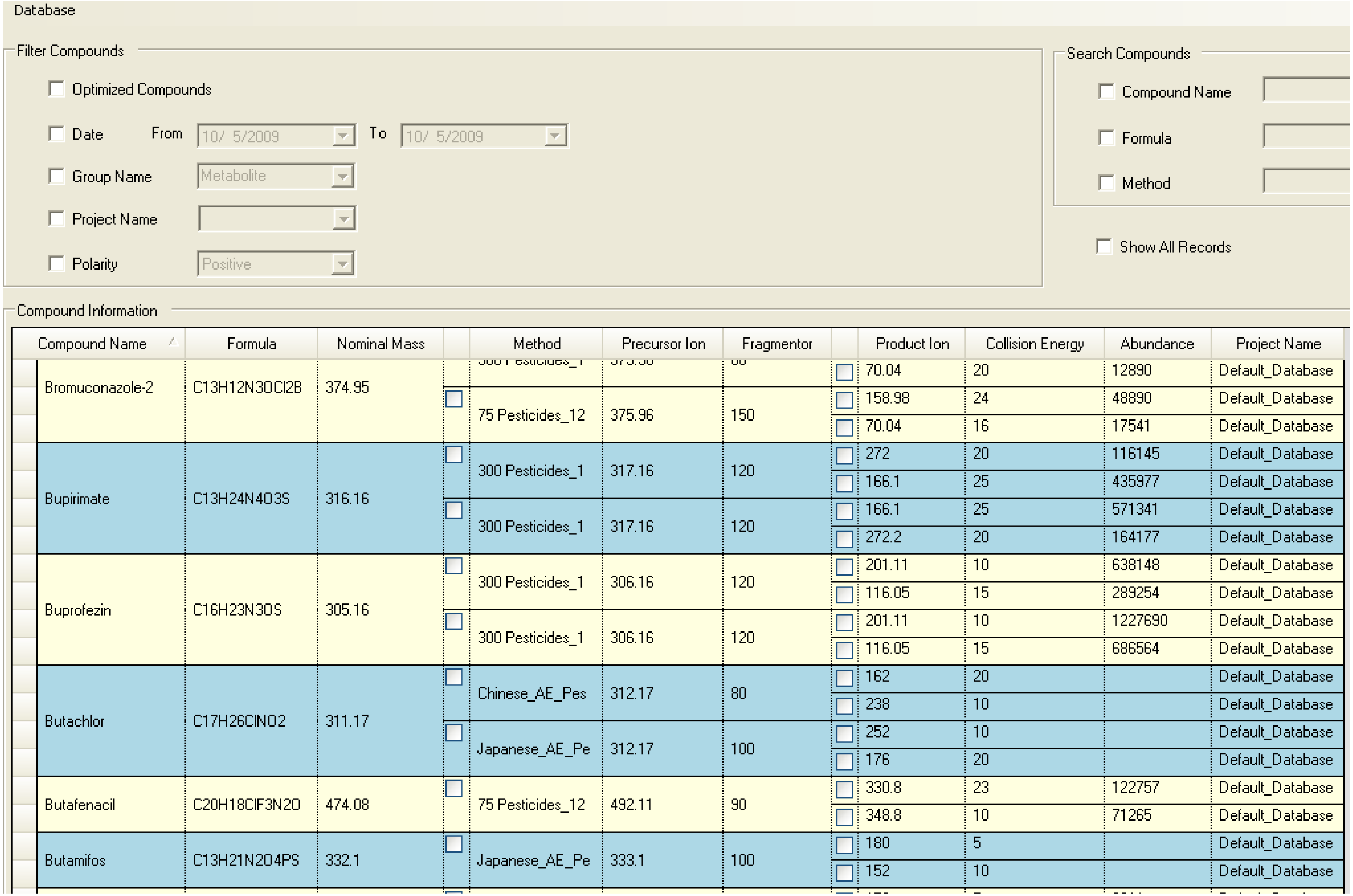
☐ Fine Step (This takes more time)

Collision Energy

Coarse From 20 To 50 Step 10

☑ Fine Step 5 (This takes more time)

MRM - Database



Database

Filter Compounds

- ☐ Optimized Compounds
- ☐ Date From 10/ 5/2009 To 10/ 5/2009
- ☐ Group Name: Metabolite
- ☐ Project Name:
- ☐ Polarity: Positive

Search Compounds

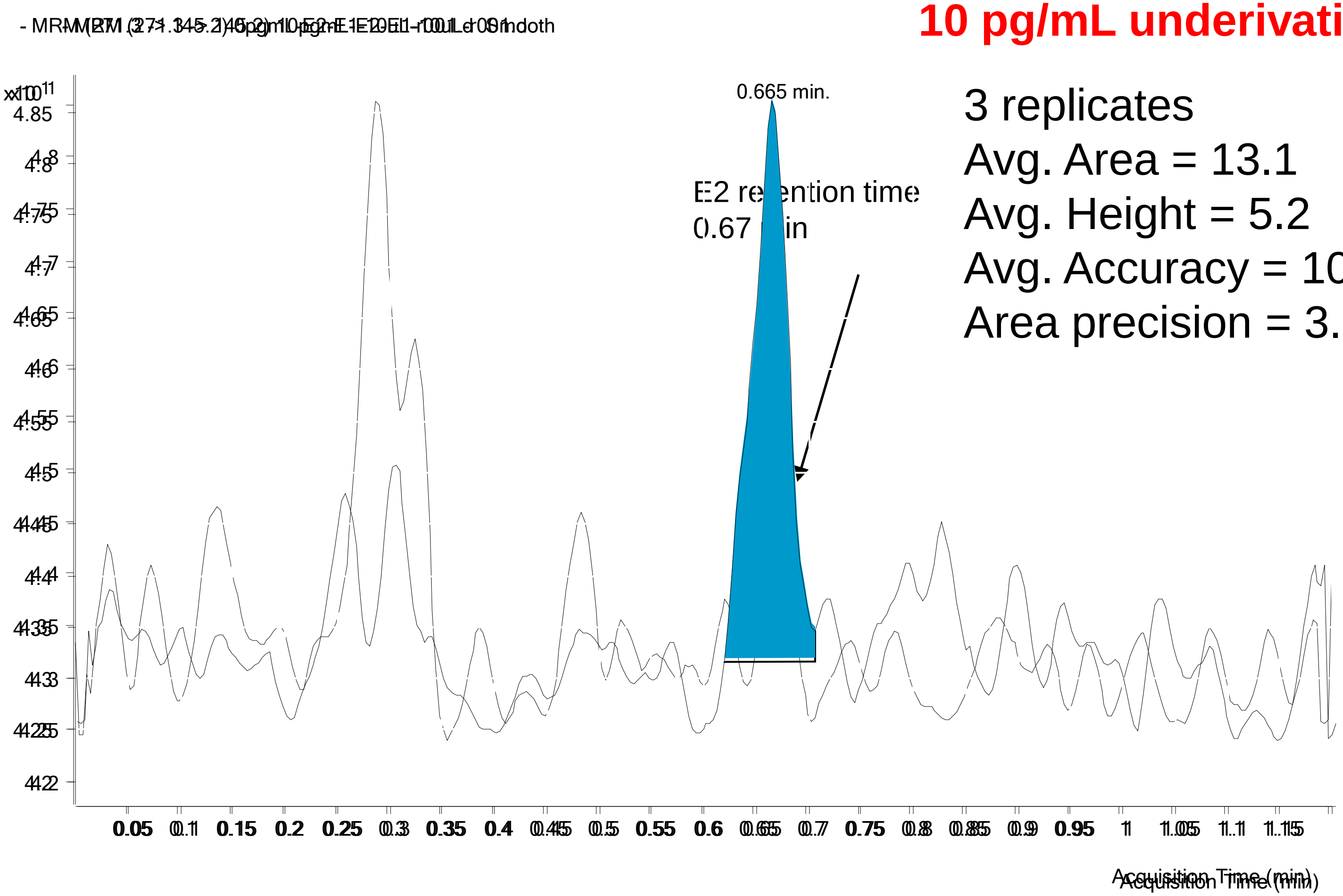
- ☐ Compound Name
- ☐ Formula
- ☐ Method

☐ Show All Records

Compound Information

Compound Name	Formula	Nominal Mass		Method	Precursor Ion	Fragmentor		Product Ion	Collision Energy	Abundance	Project Name
Bromuconazole-2	C13H12N3OCl2B	374.95					☐	70.04	20	12890	Default_Database
			☐	75 Pesticides_12	375.96	150	☐	158.98	24	48890	Default_Database
							☐	70.04	16	17541	Default_Database
Bupirimate	C13H24N4O3S	316.16	☐	300 Pesticides_1	317.16	120	☐	272	20	116145	Default_Database
							☐	166.1	25	435977	Default_Database
			☐	300 Pesticides_1	317.16	120	☐	166.1	25	571341	Default_Database
							☐	272.2	20	164177	Default_Database
Buprofezin	C16H23N3OS	305.16	☐	300 Pesticides_1	306.16	120	☐	201.11	10	638148	Default_Database
							☐	116.05	15	289254	Default_Database
			☐	300 Pesticides_1	306.16	120	☐	201.11	10	1227690	Default_Database
							☐	116.05	15	686564	Default_Database
Butachlor	C17H26ClNO2	311.17	☐	Chinese_AE_Pes	312.17	80	☐	162	20		Default_Database
							☐	238	10		Default_Database
			☐	Japanese_AE_Pe	312.17	100	☐	252	10		Default_Database
							☐	176	20		Default_Database
Butafenacil	C20H18ClF3N2O	474.08	☐	75 Pesticides_12	492.11	90	☐	330.8	23	122757	Default_Database
							☐	348.8	10	71265	Default_Database
Butamifos	C13H21N2O4PS	332.1	☐	Japanese_AE_Pe	333.1	100	☐	180	5		Default_Database
							☐	152	10		Default_Database

Quantitation of underivatized 17ß-estradiol (E2) in human plasma



10 pg/mL underivatized E2

3 replicates

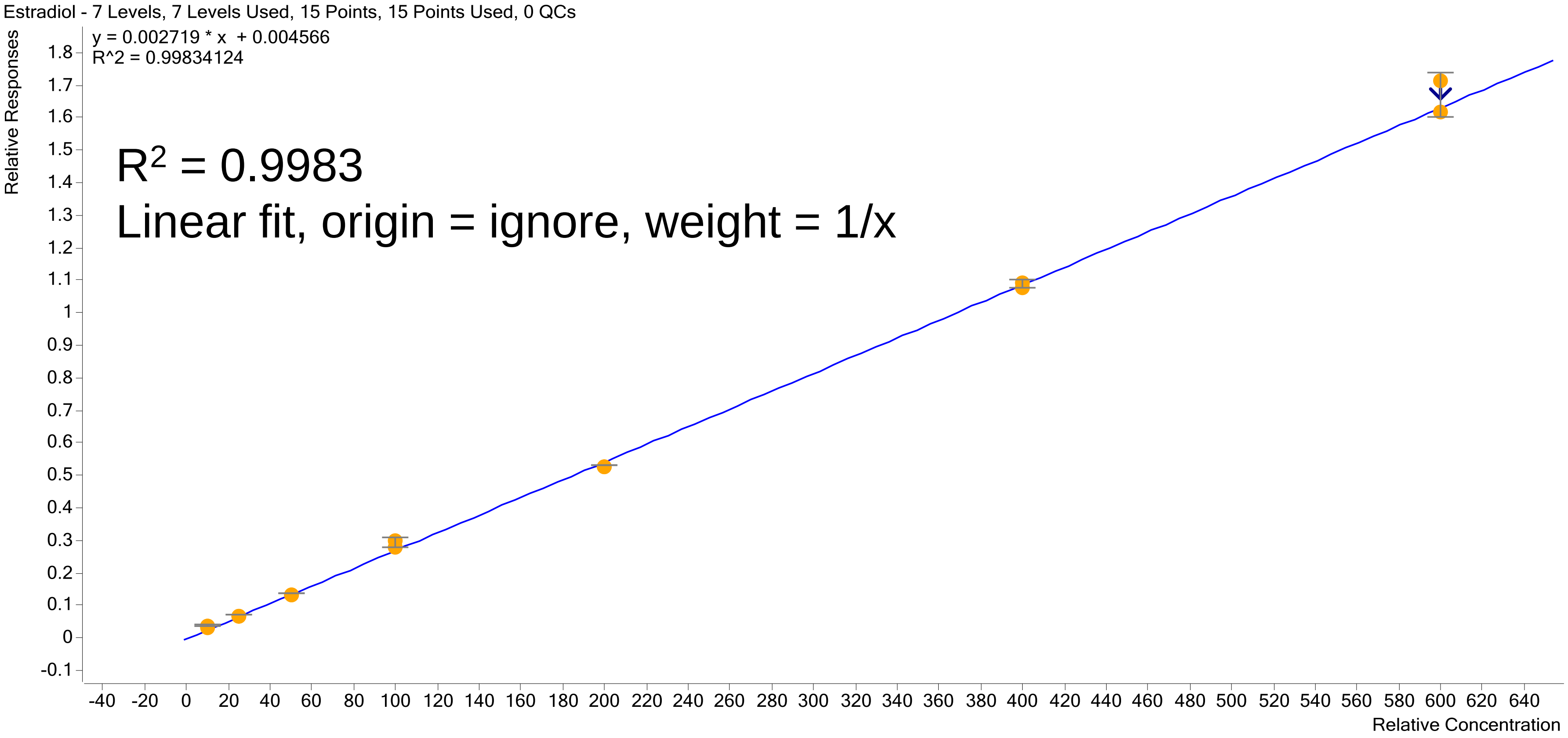
Avg. Area = 13.1

Avg. Height = 5.2

Avg. Accuracy = 108.6 %

Area precision = 3.9%

Linearity – Underivatized E2



$R^2 = 0.9983$

Linear fit, origin = ignore, weight = 1/x

Related literature

Determination of Fluticasone Proprionate in Human Plasma –

Sensitivity of Agilent 6490 Triple Quadrupole LC/MS Saves Time by Simplifying Sample Preparation

Determination of Estradiol in Human Plasma –

Agilent 6490 Triple Quadrupole LC/MS achieves low pg/mL Detection Limits without Sample Derivatization

Robustness of the 6490 Triple Quad LC/MS with iFunnel Technology for the Analysis of Verapamil in Human Plasma

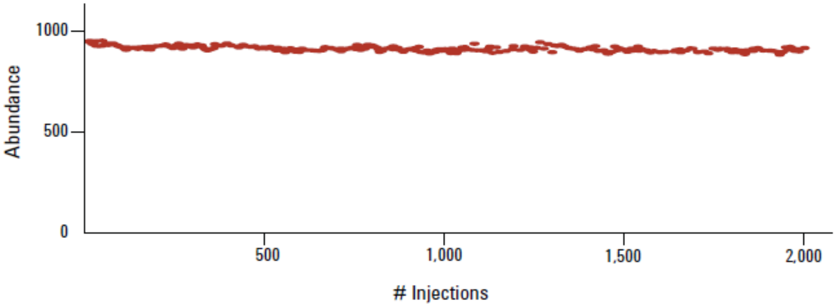
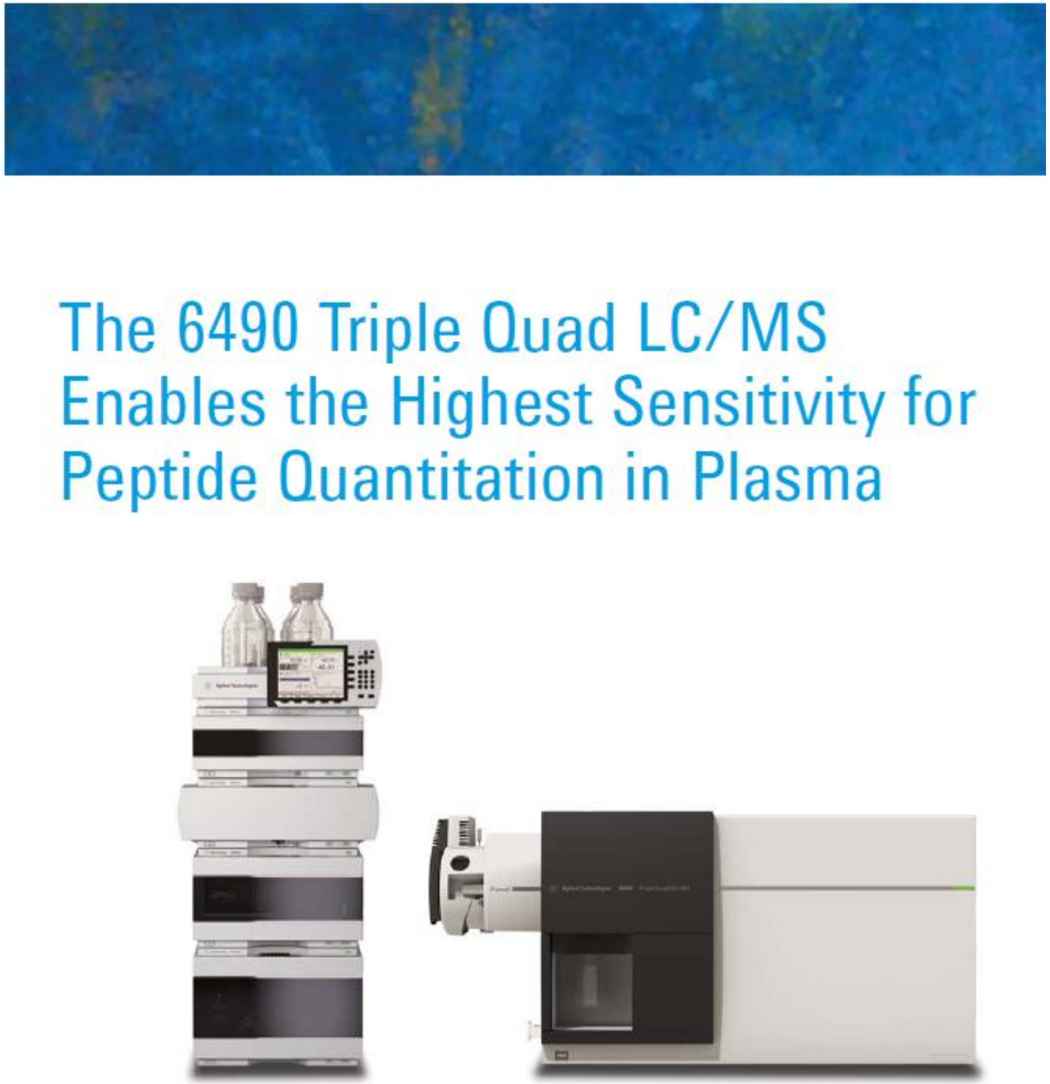


Figure 1. Absolute Area Response (2,000 injections over a period of 4 days). Area RSD = 5.3%

Peptides, proteins, oligonucleotides…

BIOPHARMA APPLICATIONS

Following from before…



The 6490 Triple Quad LC/MS Enables the Highest Sensitivity for Peptide Quantitation in Plasma

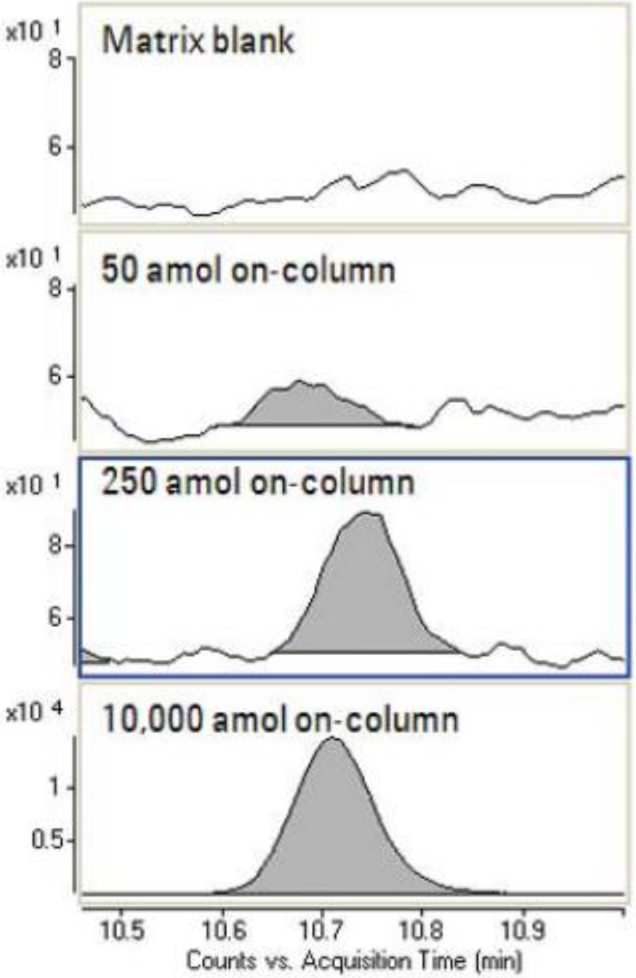
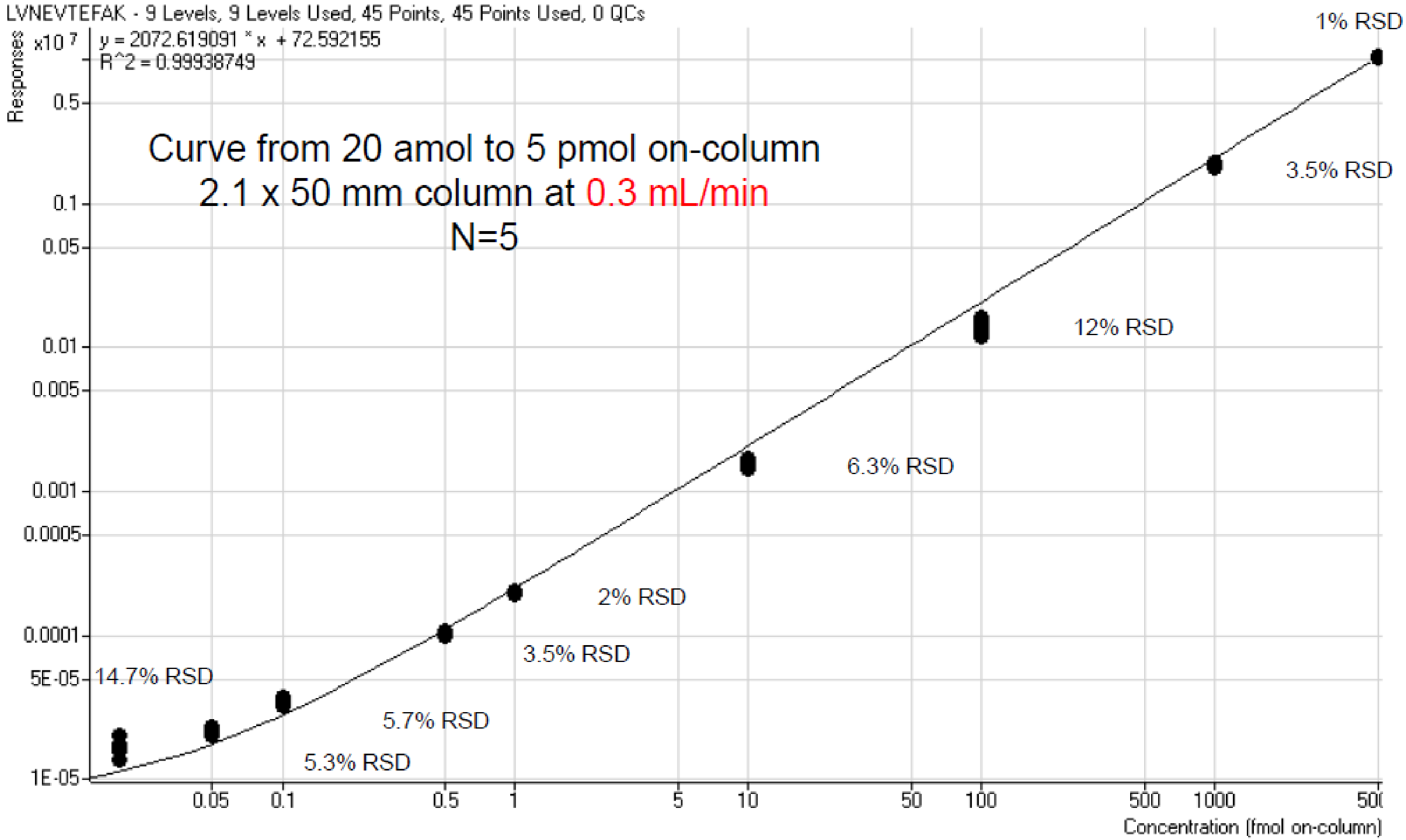


Figure 2: MRM chromatograms (441.3 → 621.4) for the plasminogen SIS peptide spiked at different levels in plasma matrix.

HSA Standard Peptide: LVNEVTEFAK
Log/Log Plot of Calibration Curve



Curve from 20 amol to 5 pmol on-column
2.1 x 50 mm column at 0.3 mL/min
N=5

1% RSD

3.5% RSD

12% RSD

6.3% RSD

2% RSD

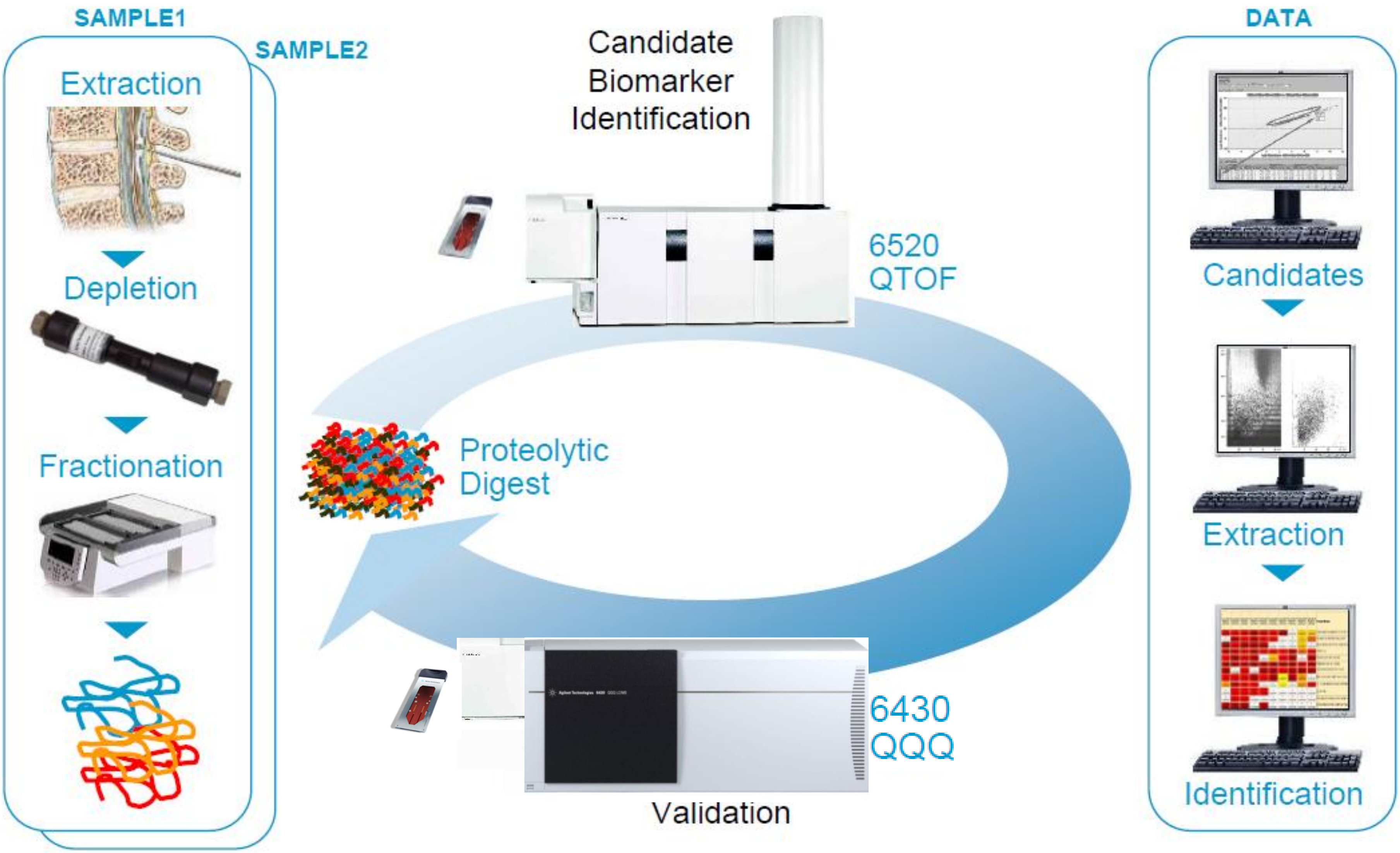
3.5% RSD

14.7% RSD

5.7% RSD

5.3% RSD

Agilent Proteomics Biomarker Workflow



SAMPLE1

SAMPLE2

Extraction

Depletion

Fractionation

Candidate
Biomarker
Identification

6520
QTOF

Proteolytic
Digest

6430
QQQ

Validation

DATA

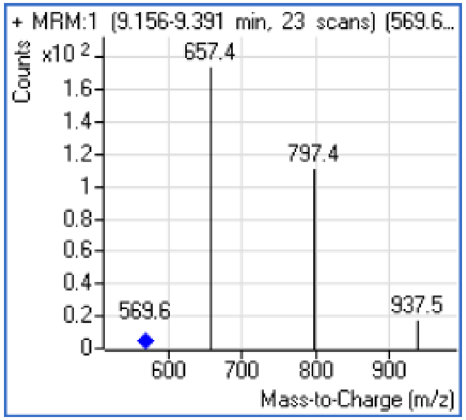
Candidates

Extraction

Identification

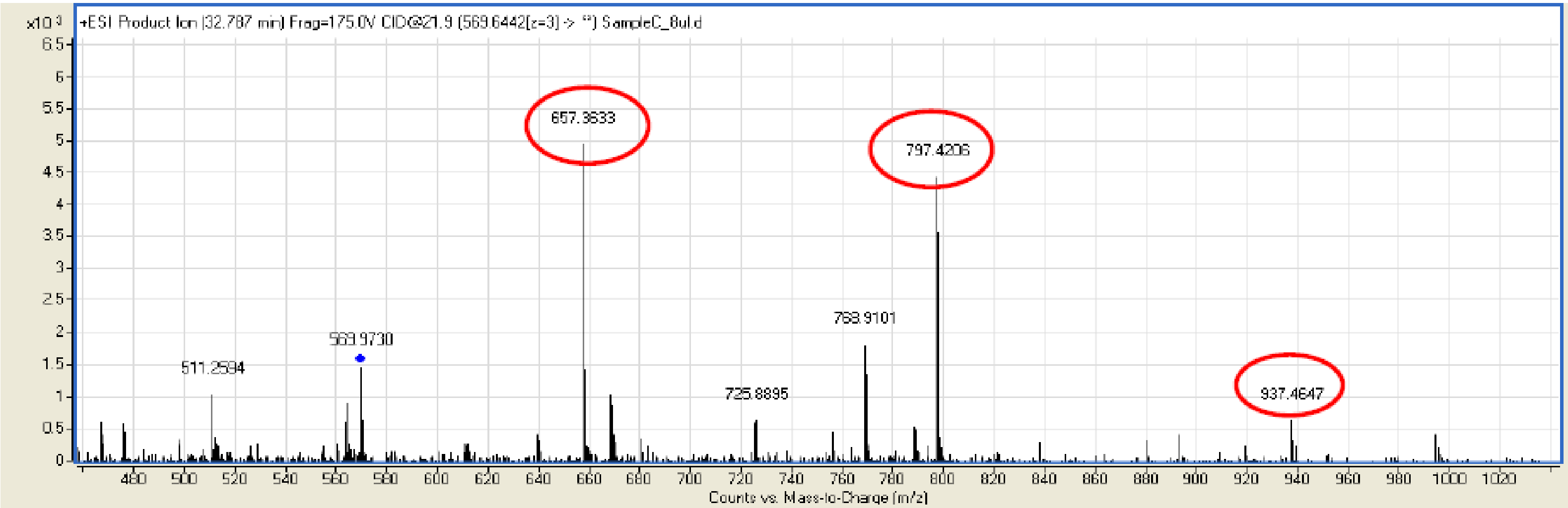
Method transfer QTOF → QQQ

Multiple MRMS

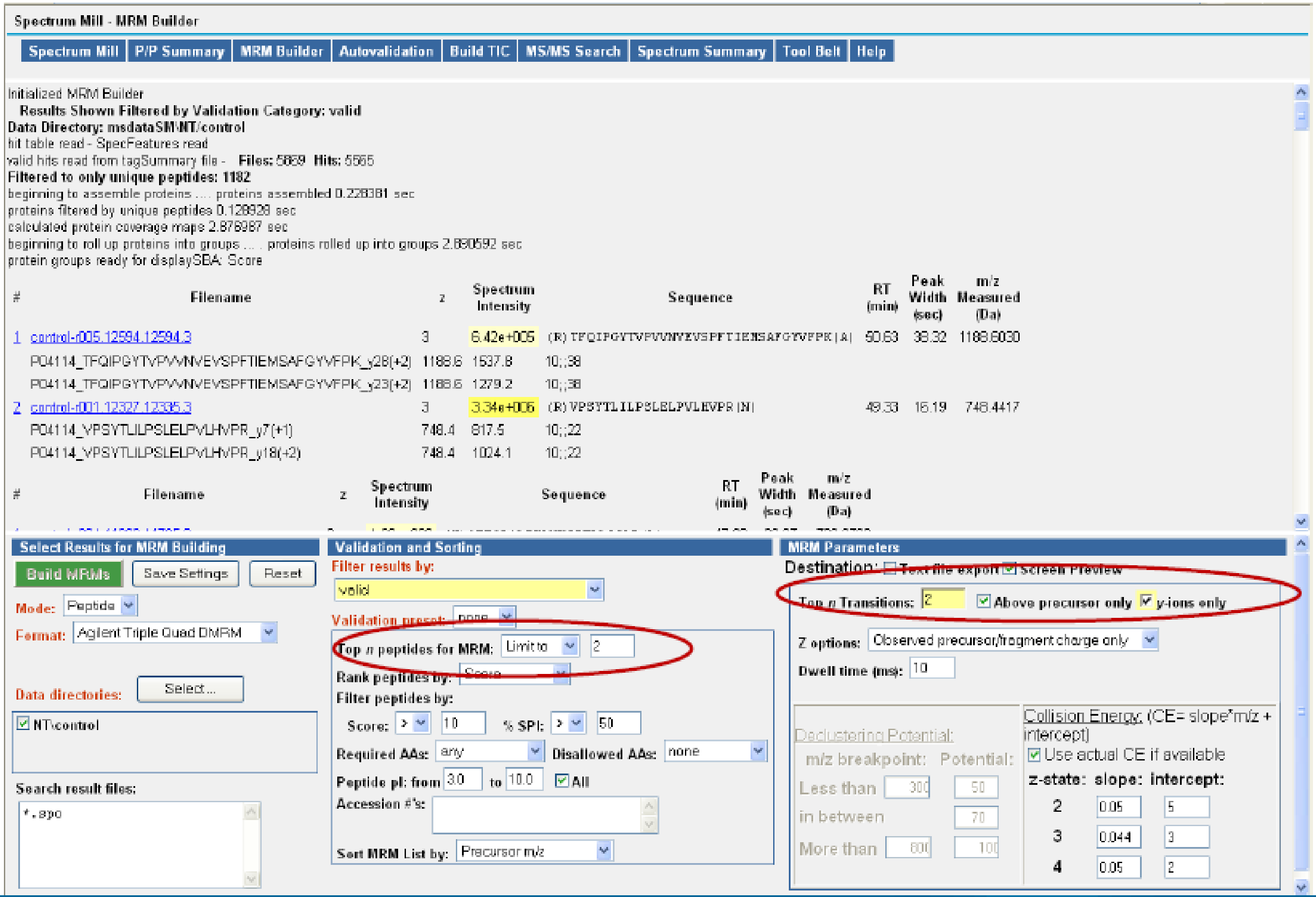


Multiple MRM data have similar relative abundance ratios as seen in the QTOF MS/MS scan. This provides confirmation of the peptides identity without having to generate an MS2 scan and with greater sensitivity

MS/MS Spectrum



MRM Builder
enerates (D)MRM method from discovery QTOF data



Initialized MRM Builder
Results Shown Filtered by Validation Category: valid
Data Directory: msdataSM\NT/control
hit table read - SpecFeatures read
valid hits read from tagSummary file - **Files:** 5869 **Hits:** 5565
Filtered to only unique peptides: 1182
beginning to assemble proteins .... proteins assembled 0.228381 sec
proteins filtered by unique peptides 0.128928 sec
calculated protein coverage maps 2.876987 sec
beginning to roll up proteins into groups .... proteins rolled up into groups 2.830592 sec
protein groups ready for displaySBA: Score

#	Filename	z	Spectrum Intensity	Sequence	RT (min)	Peak Width (sec)	m/z Measured (Da)
1	control-r005.12594.12594.3	3	6.42e+005	(R)TFQIPGYTVPVVNVEVSPFTIEMSAFGYVFPK(A)	50.63	38.32	1188.6030
	P04114_TFQIPGYTVPVVNVEVSPFTIEMSAFGYVFPK_y28(+2)	1188.6	1537.8	10;;38			
	P04114_TFQIPGYTVPVVNVEVSPFTIEMSAFGYVFPK_y23(+2)	1188.6	1279.2	10;;38			
2	control-r001.12327.12335.3	3	3.34e+006	(R)VPSYTLILPSLELPVLHVPR(N)	49.33	16.19	748.4417
	P04114_VPSYTLILPSLELPVLHVPR_y7(+1)	748.4	817.5	10;;22			
	P04114_VPSYTLILPSLELPVLHVPR_y18(+2)	748.4	1024.1	10;;22			

#	Filename	z	Spectrum Intensity	Sequence	RT (min)	Peak Width (sec)	m/z Measured (Da)

Select Results for MRM Building

Mode: Peptide
Format: Agilent Triple Quad DMRM
Data directories:
☑ NT\control
Search result files:
*.spo

Validation and Sorting

Filter results by: valid
Validation preset: none
Top *n* peptides for MRM: Limit to 2
Rank peptides by: Score
Filter peptides by:
Score: > 10 % SPI: > 50
Required AAs: any Disallowed AAs: none
Peptide pI: from 3.0 to 10.0 ☑ All
Accession #'s:
Sort MRM List by: Precursor m/z

MRM Parameters

Destination: ☐ Text file export ☑ Screen Preview
Top *n* Transitions: 2 ☑ Above precursor only ☑ y-ions only
Z options: Observed precursor/fragment charge only
Dwell time (ms): 10

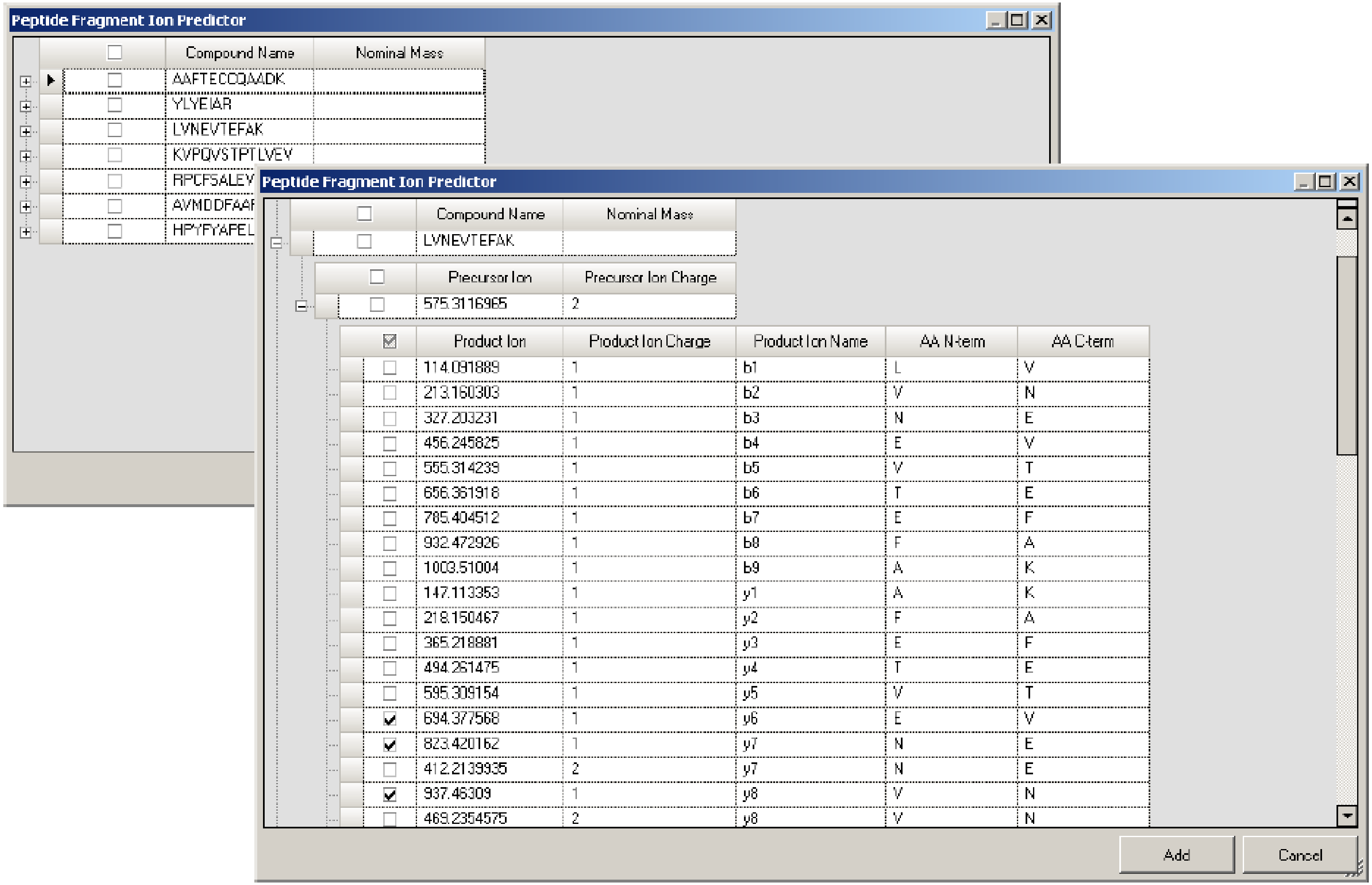
Declustering Potential:

m/z breakpoint:		Potential:
Less than	300	50
in between		70
More than	800	100

Collision Energy: (CE= slope*m/z + intercept)
☑ Use actual CE if available

z-state:	slope:	intercept:
2	0.05	5
3	0.044	3
4	0.05	2

Enter sequences and select b/y ions for optimization



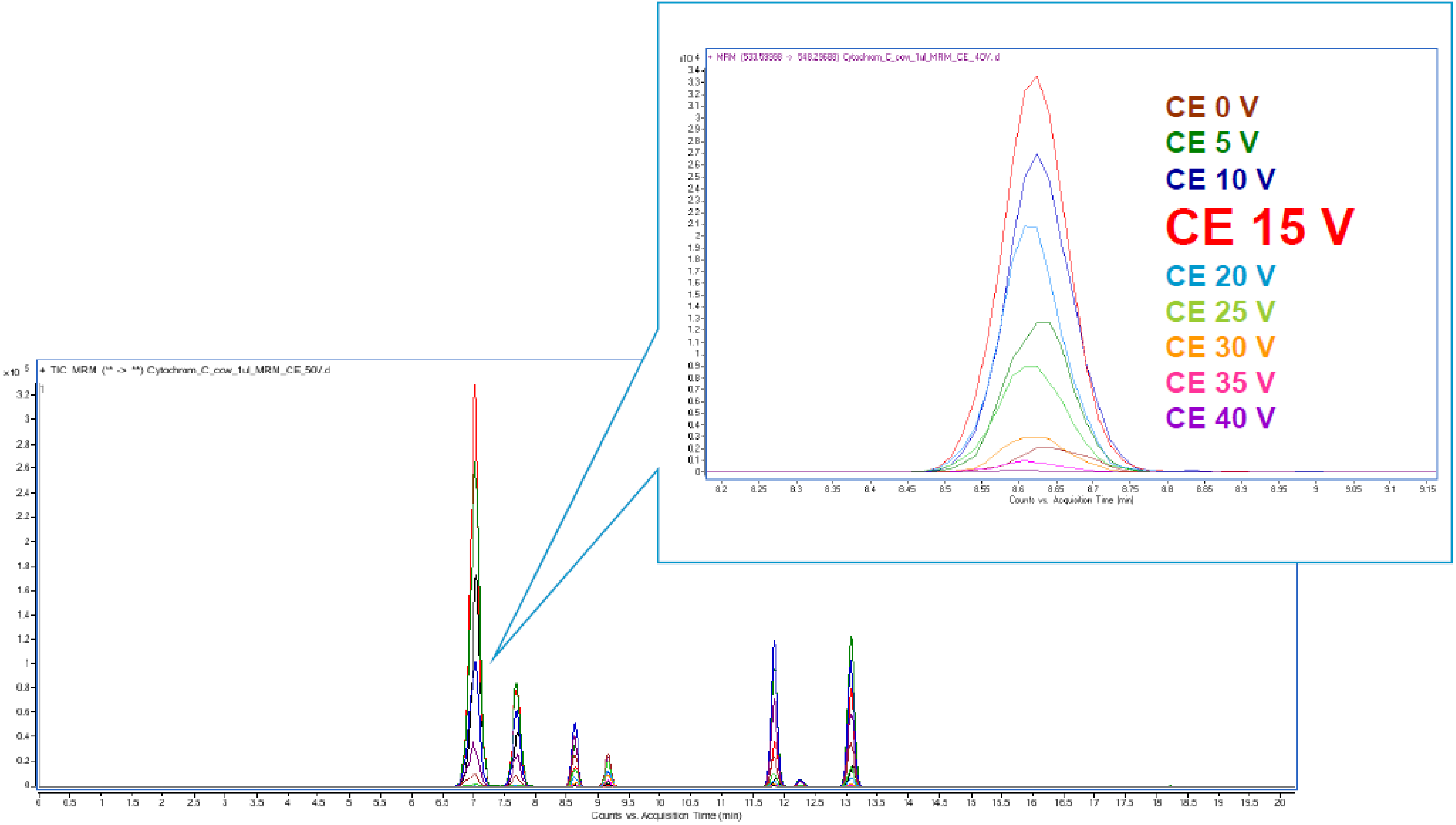
☐	Compound Name	Nominal Mass
☐	AAFTECCQAADK	
☐	YLYEIAR	
☐	LVNEVTEFAK	
☐	KVPQVSTPTLVEV	
☐	RPCFSALEV	
☐	AVMDDFAAF	
☐	HPYFYAPEL	

☐	Compound Name	Nominal Mass
☐	LVNEVTEFAK	

☐	Precursor Ion	Precursor Ion Charge
☐	575.3116965	2

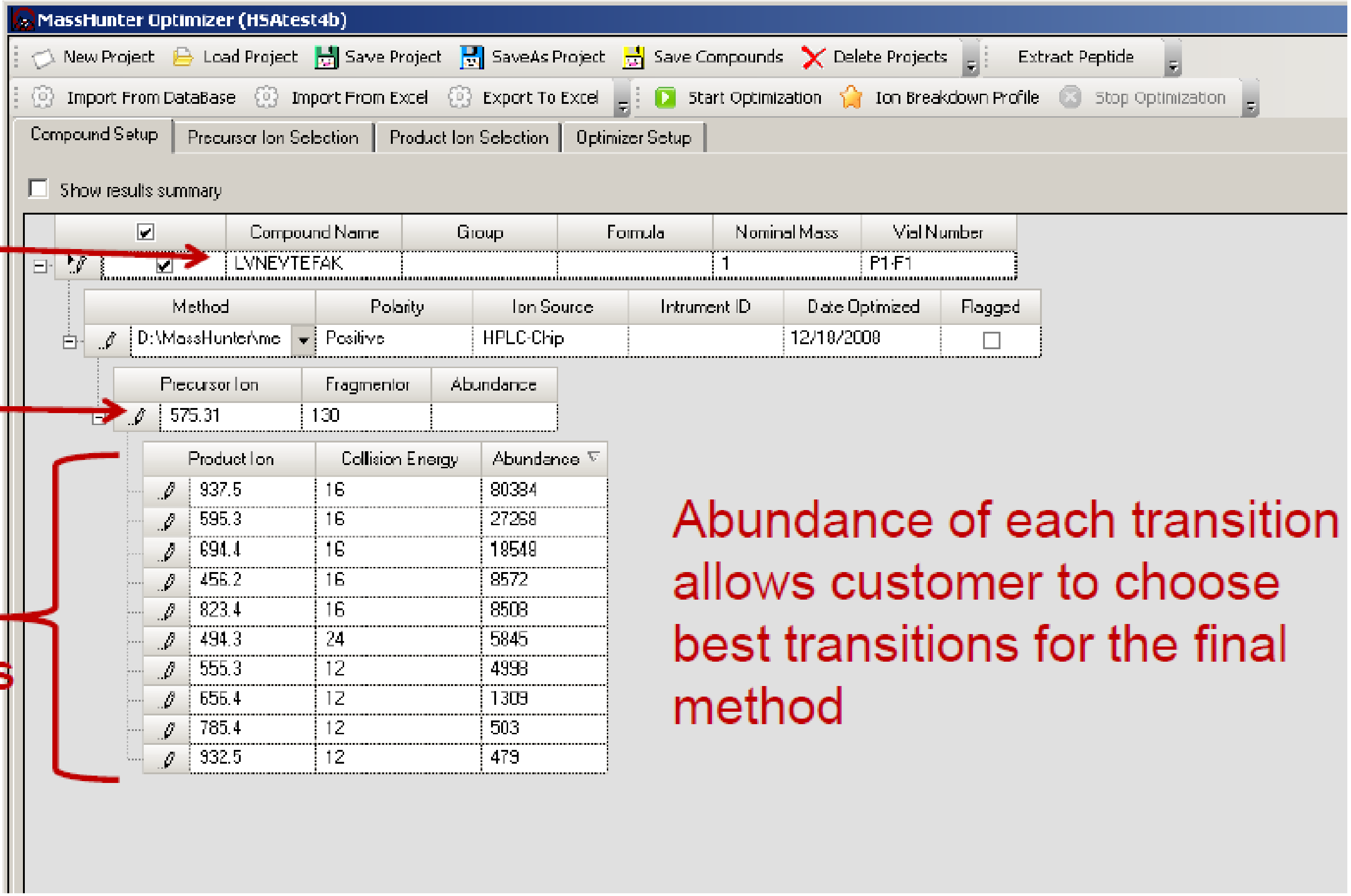
☑	Product Ion	Product Ion Charge	Product Ion Name	AA N-term	AA C-term
☐	114.091889	1	b1	L	V
☐	213.160303	1	b2	V	N
☐	327.203231	1	b3	N	E
☐	456.245825	1	b4	E	V
☐	555.314239	1	b5	V	T
☐	656.361918	1	b6	T	E
☐	785.404512	1	b7	E	F
☐	932.472926	1	b8	F	A
☐	1003.51004	1	b9	A	K
☐	147.113353	1	y1	A	K
☐	218.150467	1	y2	F	A
☐	365.218881	1	y3	E	F
☐	494.261475	1	y4	T	E
☐	595.309154	1	y5	V	T
☑	694.377568	1	y6	E	V
☑	823.420162	1	y7	N	E
☐	412.2139935	2	y7	N	E
☑	937.46309	1	y8	V	N
☐	469.2354575	2	y8	V	N

Automatic optimization of collision energies for predicted MRM transistions



CE 0 V
CE 5 V
CE 10 V
CE 15 V
CE 20 V
CE 25 V
CE 30 V
CE 35 V
CE 40 V

Results of peptide optimization



☐ Show results summary

☑	Compound Name	Group	Formula	Nominal Mass	Vial Number
☑	LVNEYTEFAK			1	P1-F1

Method	Polarity	Ion Source	Intrument ID	Date Optimized	Flagged
D:\MassHunter\me	Positive	HPLC-Chip		12/18/2008	☐

Precursor Ion	Fragmentor	Abundance
575.31	130	

Product Ion	Collision Energy	Abundance
937.5	16	80384
595.3	16	27268
694.4	16	18549
456.2	16	8572
823.4	16	8508
494.3	24	5845
555.3	12	4998
656.4	12	1309
785.4	12	503
932.5	12	479

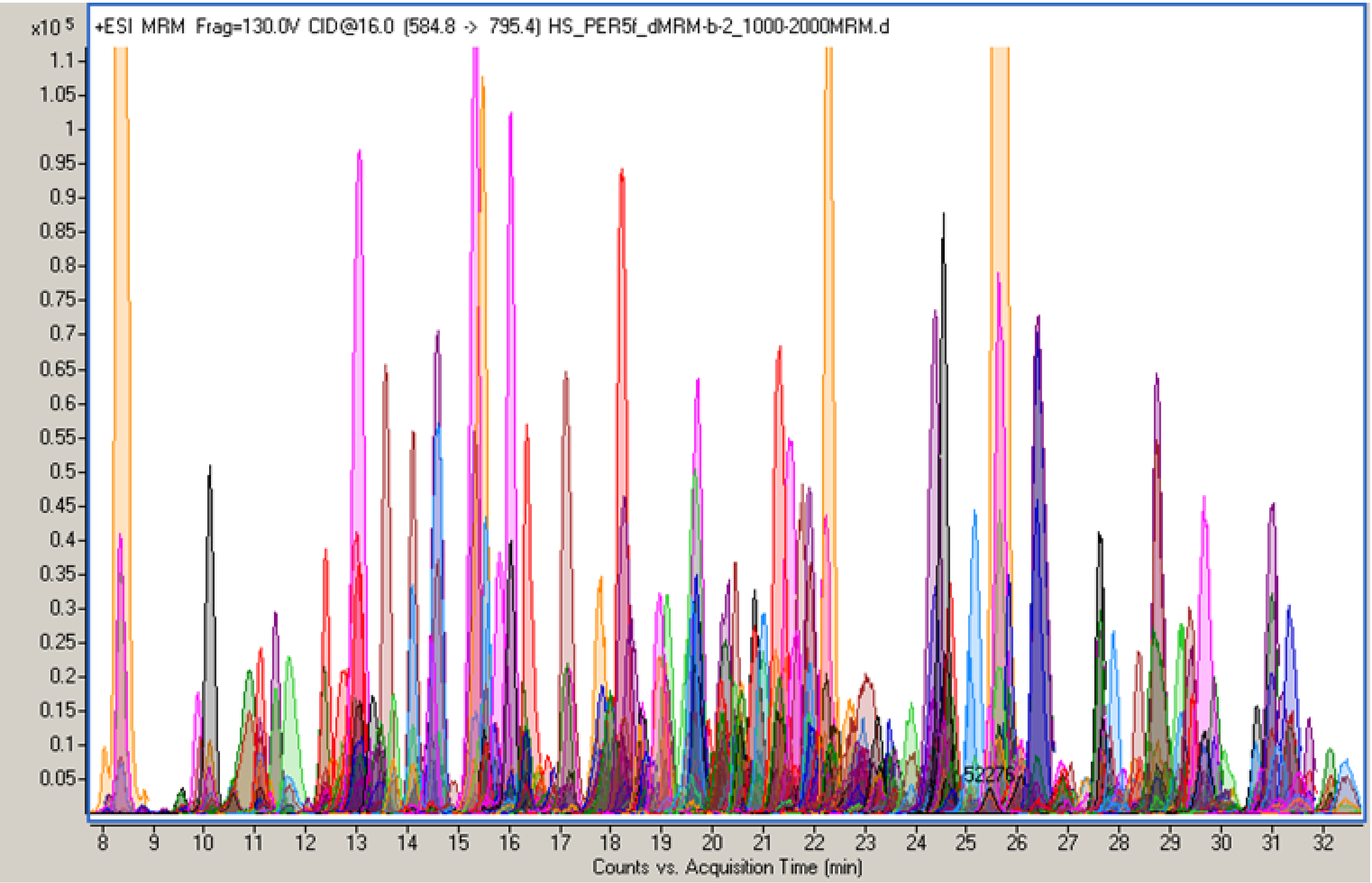
Peptide sequence

Precursor

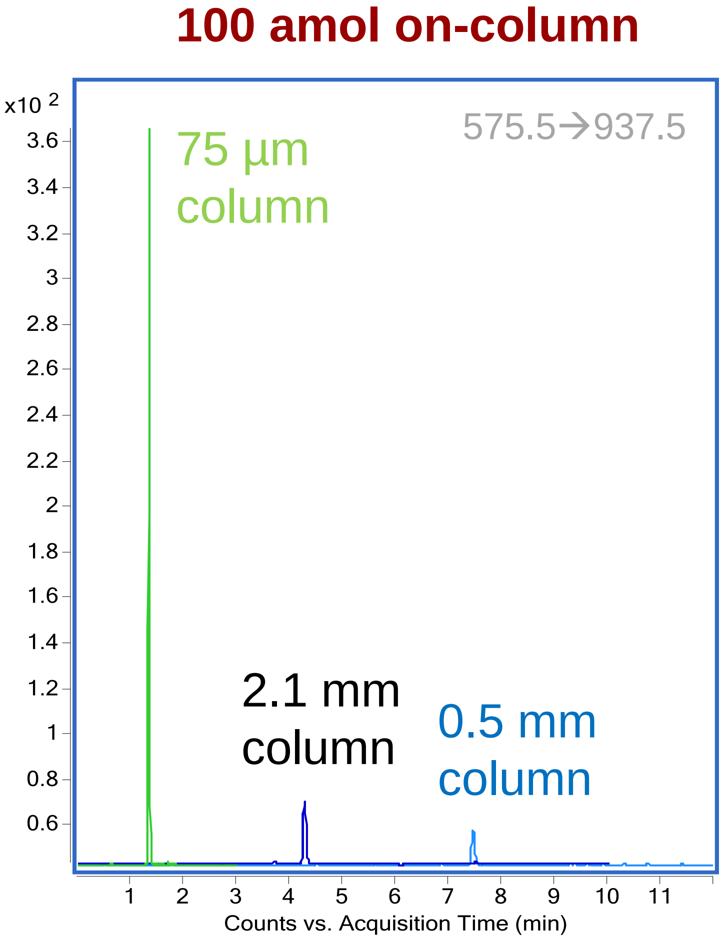
Optimized product ions

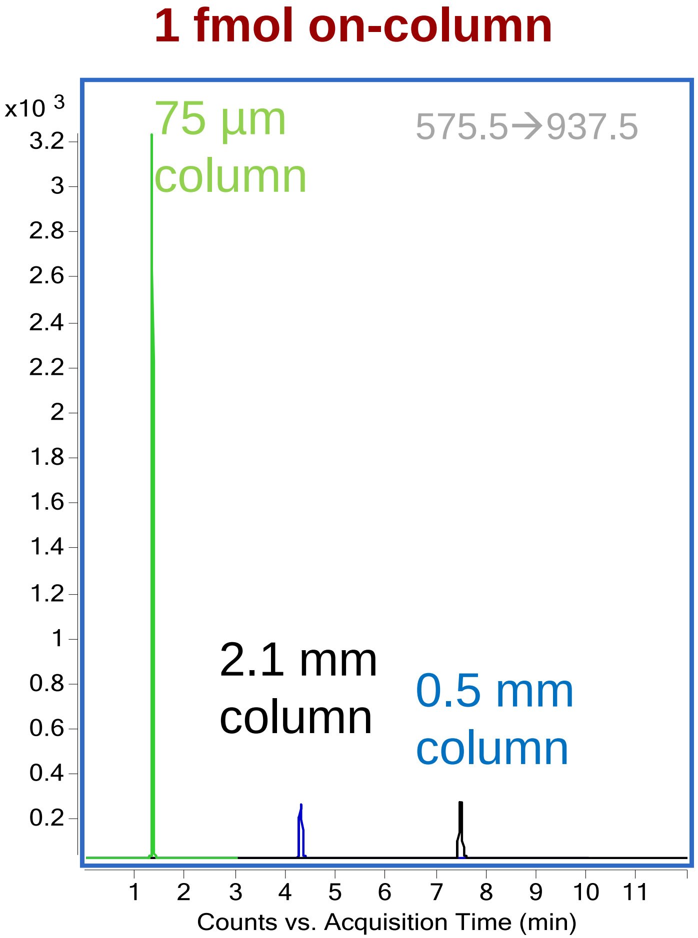
Abundance of each transition allows customer to choose best transitions for the final method

2,000 Dynamic MRM transitions in digest of depleted human sera – no time segment boundaries



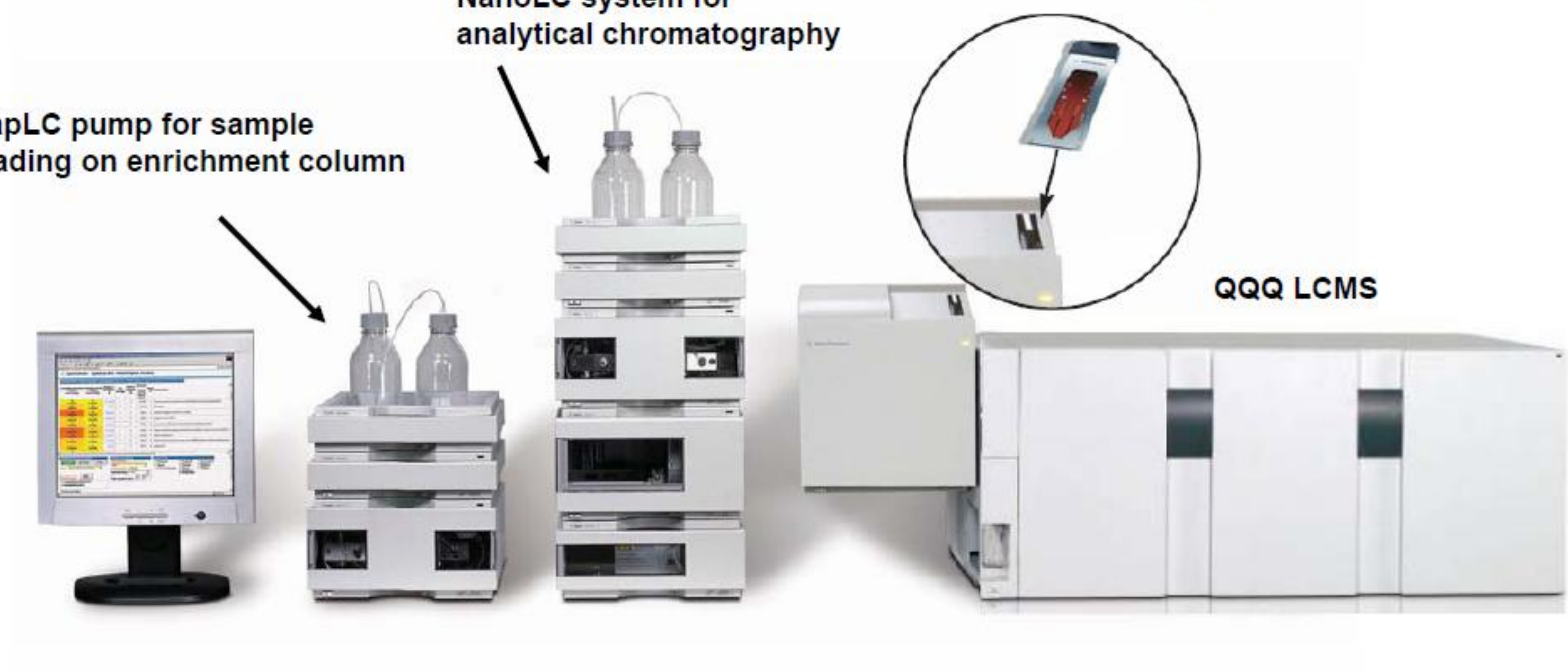
nanoLC for extra sensitivity





Agilent HPLC-Chip/QQQ LCMS Technology

Nanospray chip configuration brings new era in high sensitivity quantitation



HPLC Chip Cube system

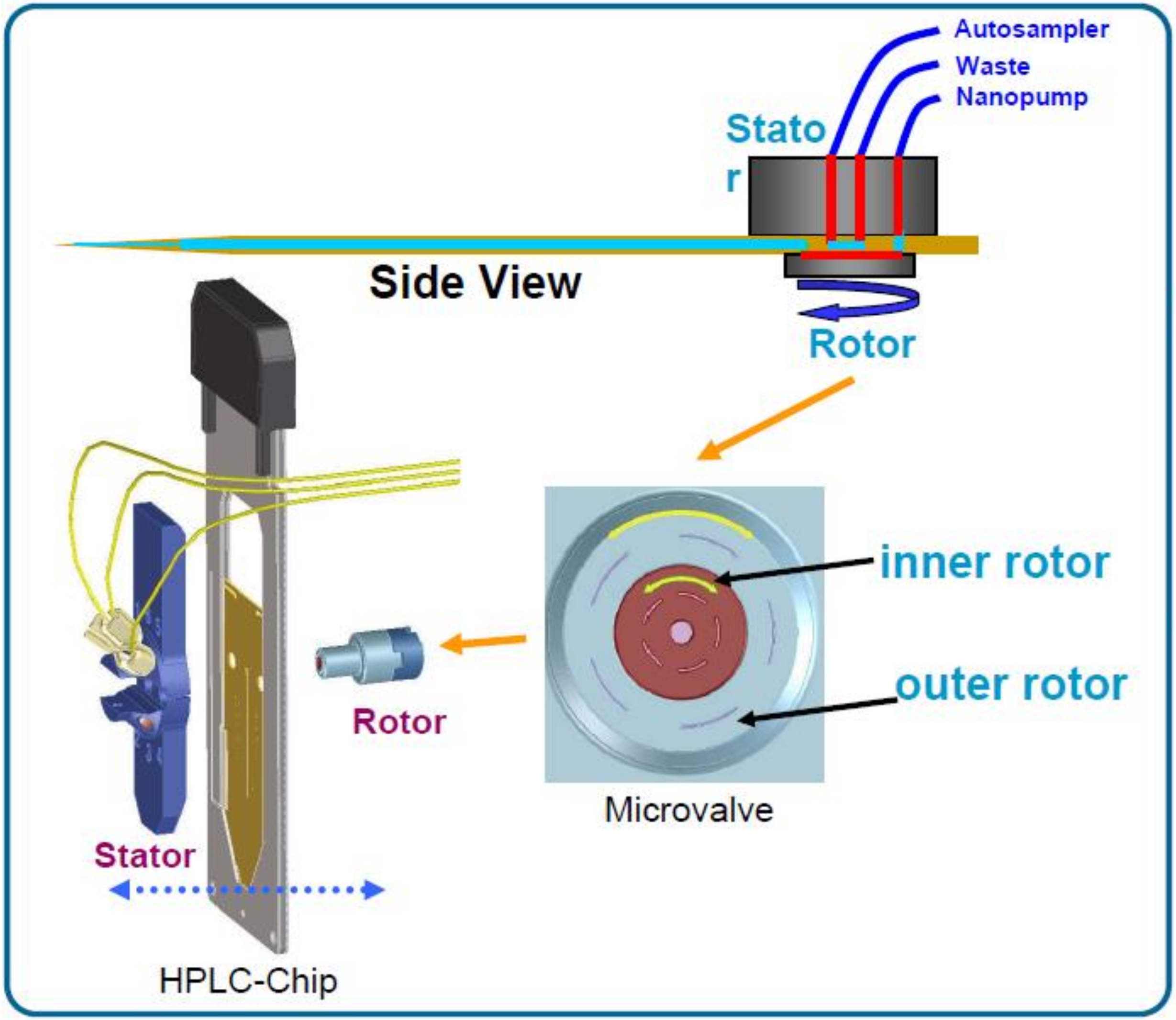
NanoLC system for
analytical chromatography

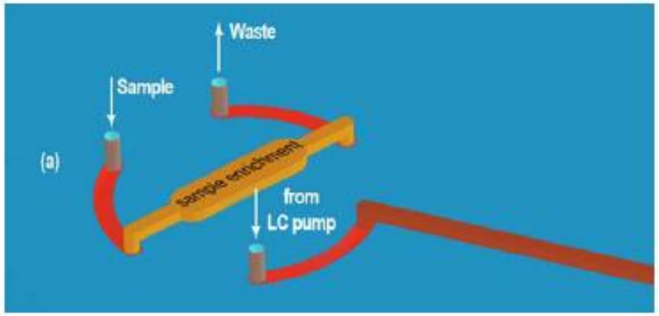
CapLC pump for sample
loading on enrichment column

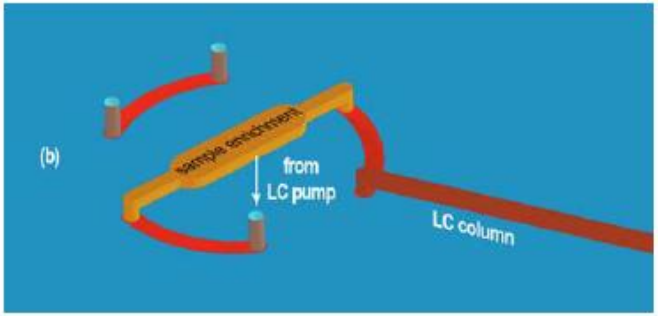
QQQ LCMS

Sensitivity: down to low amol
Dynamic range: up to 10^5

HPLC-Chip/MS Interface: Fluid Connections to the HPLC-Chip

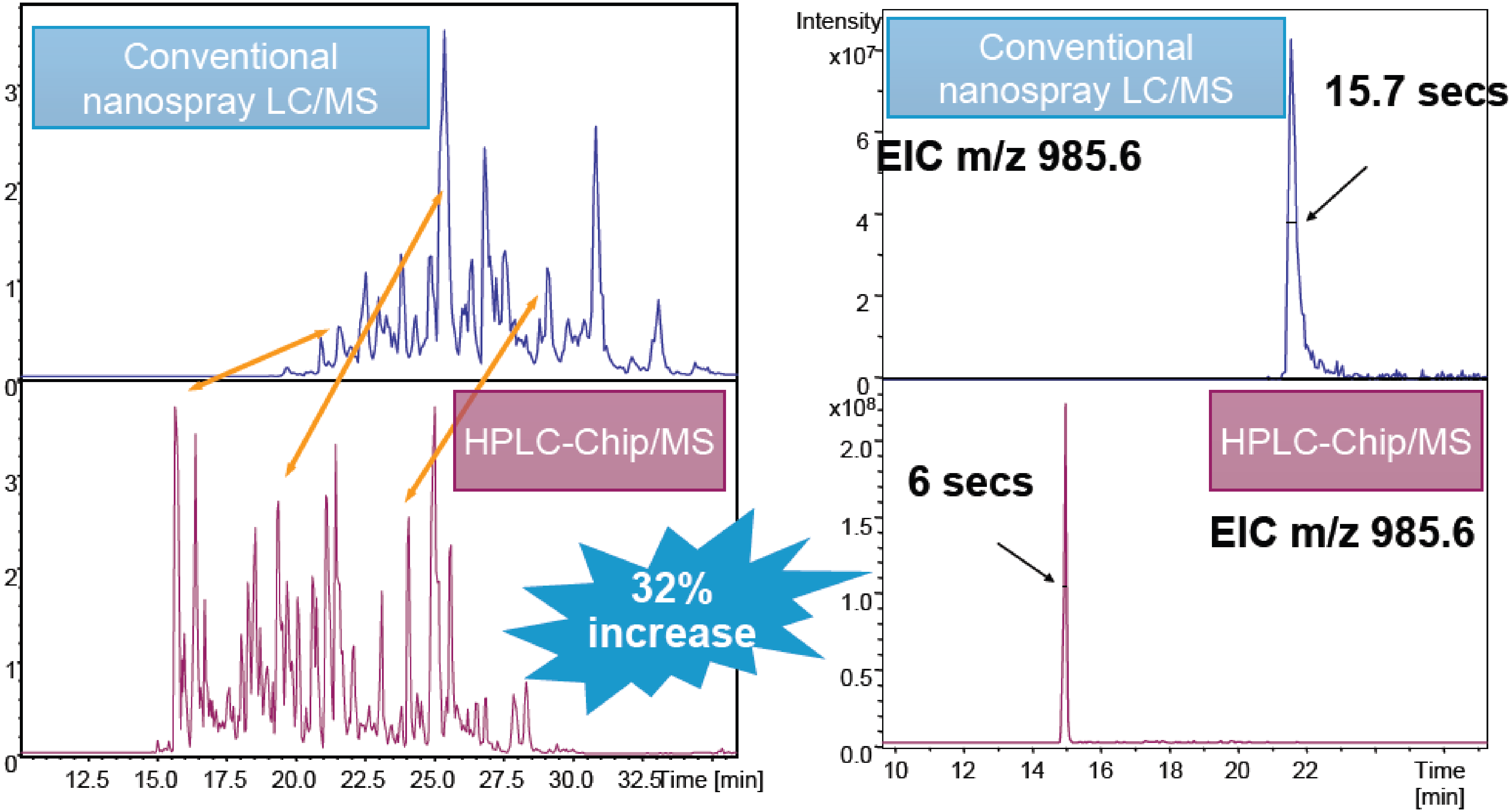

Autosampler
Waste
Nanopump
Stator
Side View
Rotor
inner rotor
outer rotor
Microvalve
Rotor
Stator
HPLC-Chip





Chromatographic Performance

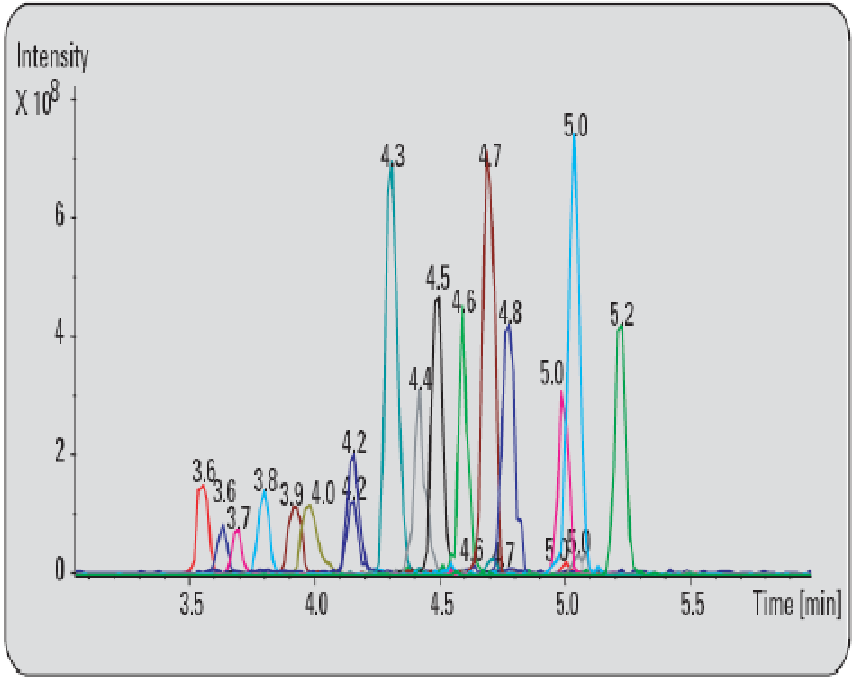
Protein Digest Mixture



Reduced MS complexity + reduced ionization competition = improved ID

Agilent Technologies

Retention Time Reproducibility

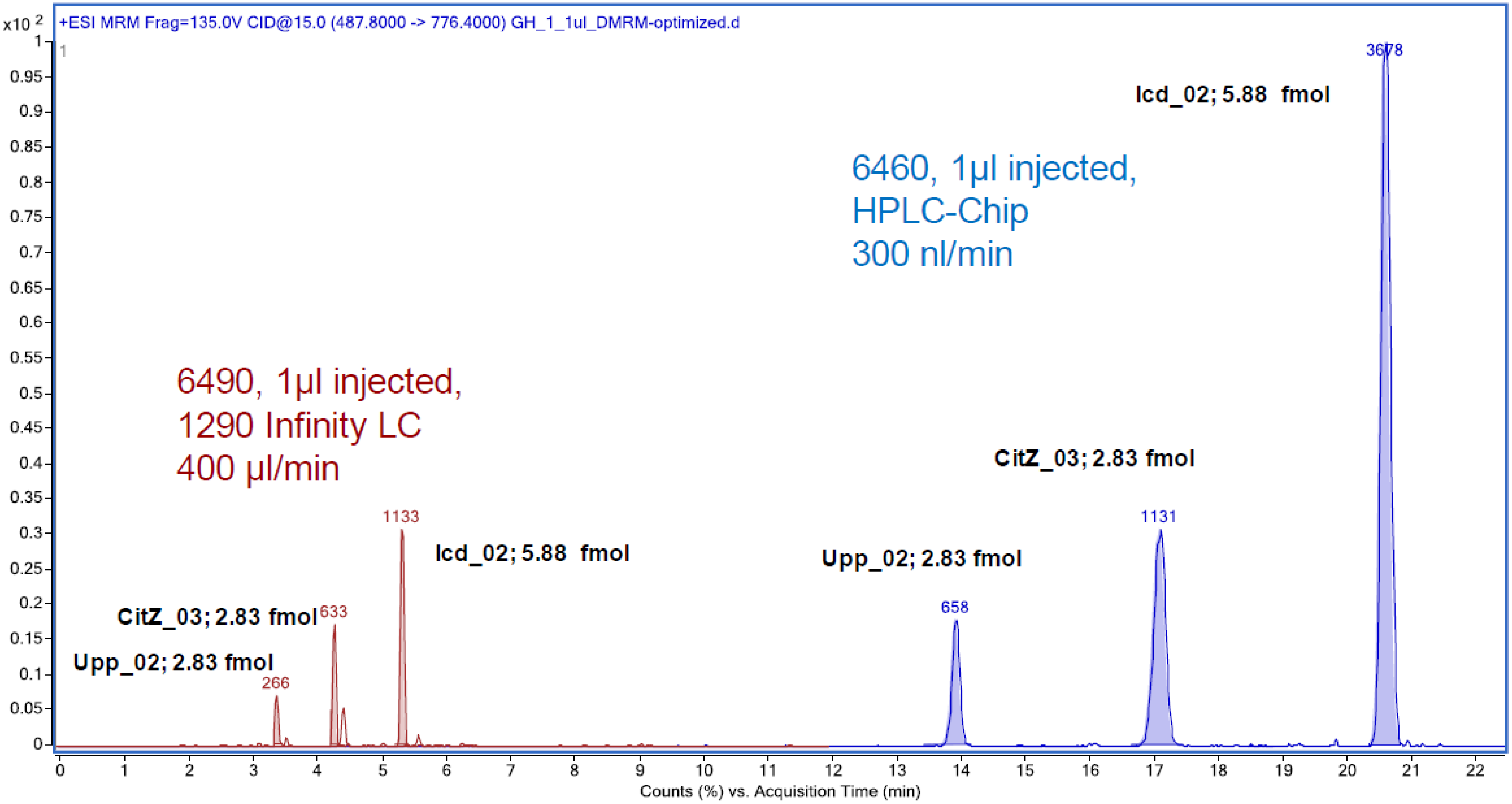


Extracted ion chromatograms for 17 peaks from a BSA tryptic digest (50 fmol on-column)

	RT	SD	%RSD
EIC 487.8	3.618	0.014	0.40
EIC 752	3.788	0.011	0.29
EIC 740.6	5.018	0.010	0.20
EIC 874.4	3.968	0.012	0.31
EIC 653.6	4.289	0.012	0.28
EIC 511.7	3.681	0.012	0.31
EIC 722.7	3.547	0.012	0.35
EIC 778	4.143	0.010	0.23
EIC 526.3	4.399	0.015	0.34
EIC 547.5	4.472	0.011	0.25
EIC 746.7	5.196	0.011	0.20
EIC 519.1	4.142	0.011	0.26
EIC 508.2	4.972	0.011	0.23
EIC 582.4	4.679	0.011	0.23
EIC 461.9	3.905	0.012	0.30
EIC 474	4.759	0.011	0.22
EIC 628	4.584	0.010	0.22

RT reproducibility evaluated using 69 repeat injections

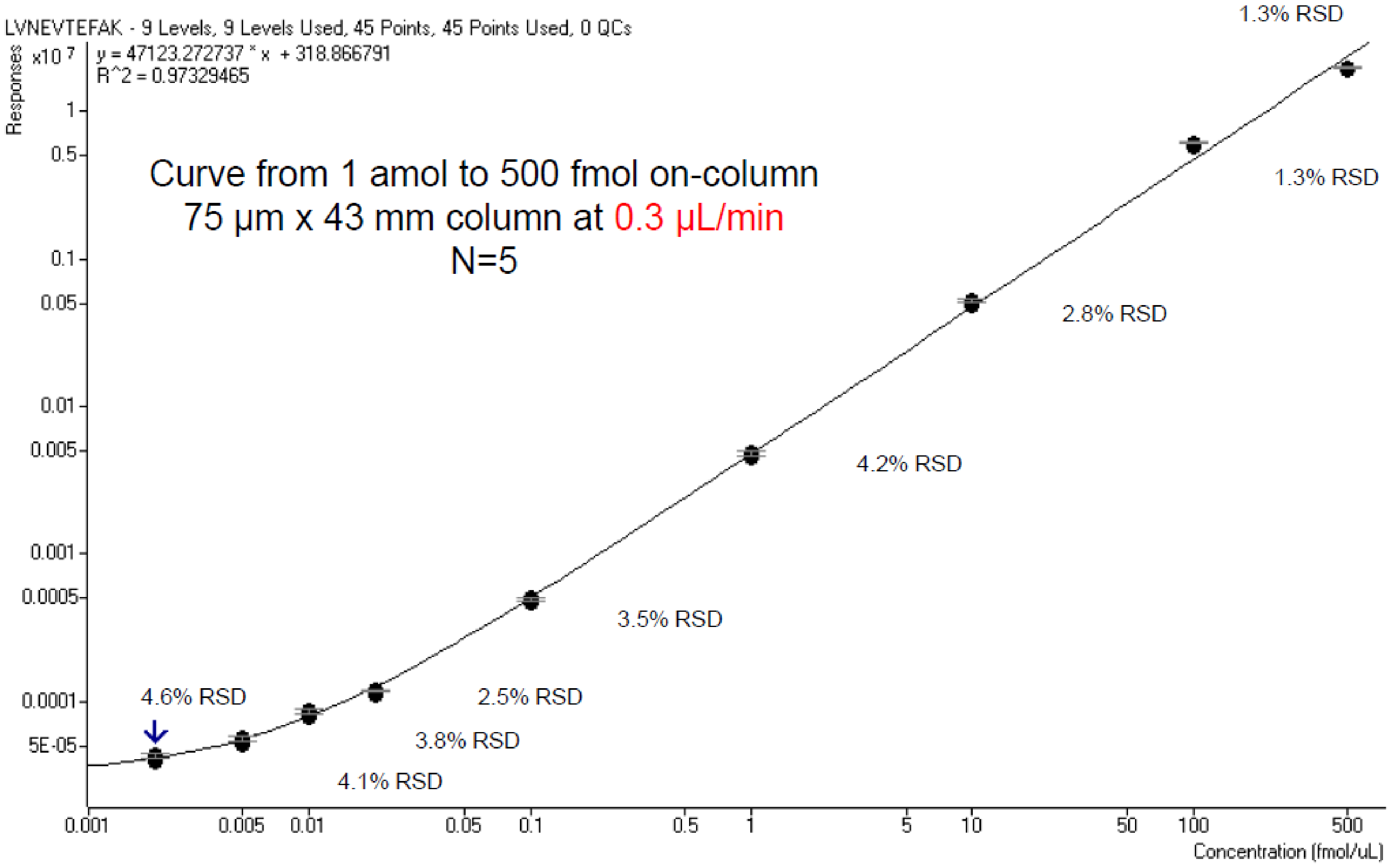
6490 QQQ sensitivity



1290/6490 → HPLC-Chip/6460: approx. 2 – 3 x improvement in signal intensity

Agilent Technologies

HSA Standard Peptide: LVNEVTEFAK
Log/Log Plot of Calibration Curve



Curve from 1 amol to 500 fmol on-column
75 µm x 43 mm column at 0.3 µL/min
N=5

Summary

- 1290 + 6490 Agilent system solutions offers the most robust, sensitive LC-QQQ on the market
  - 50 fg reserpine, 900:1 s/n
  - iFunnel Technology for improved robustness
  - QuanOptimizer and Database Manager for easy compound setup

- HPLC- Chip and 6400 series Triple Quadrupole MS/MS provides high sensitivity and large dynamic range
  - Robust and stable nanoflow with HPLC-Chip
  - Good retention time and MS detection reproducibility
  - Peptide Optimizer for maximum tune sensitivity
  - Dynamic MRM for max sensitivity, minimum cycle time