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LDTD and High Resolution Mass Spectrometry Workflows to Enhance Sample Throughput for the Automated In-Vitro ADME Laboratory

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MSACL Workshop

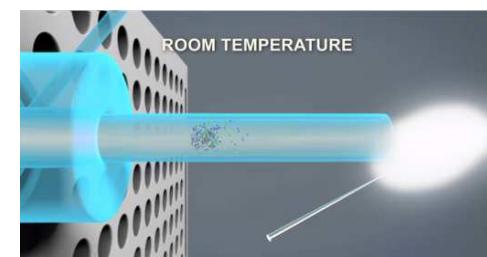
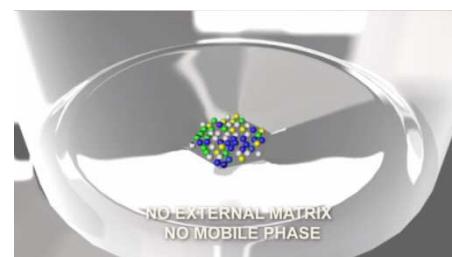
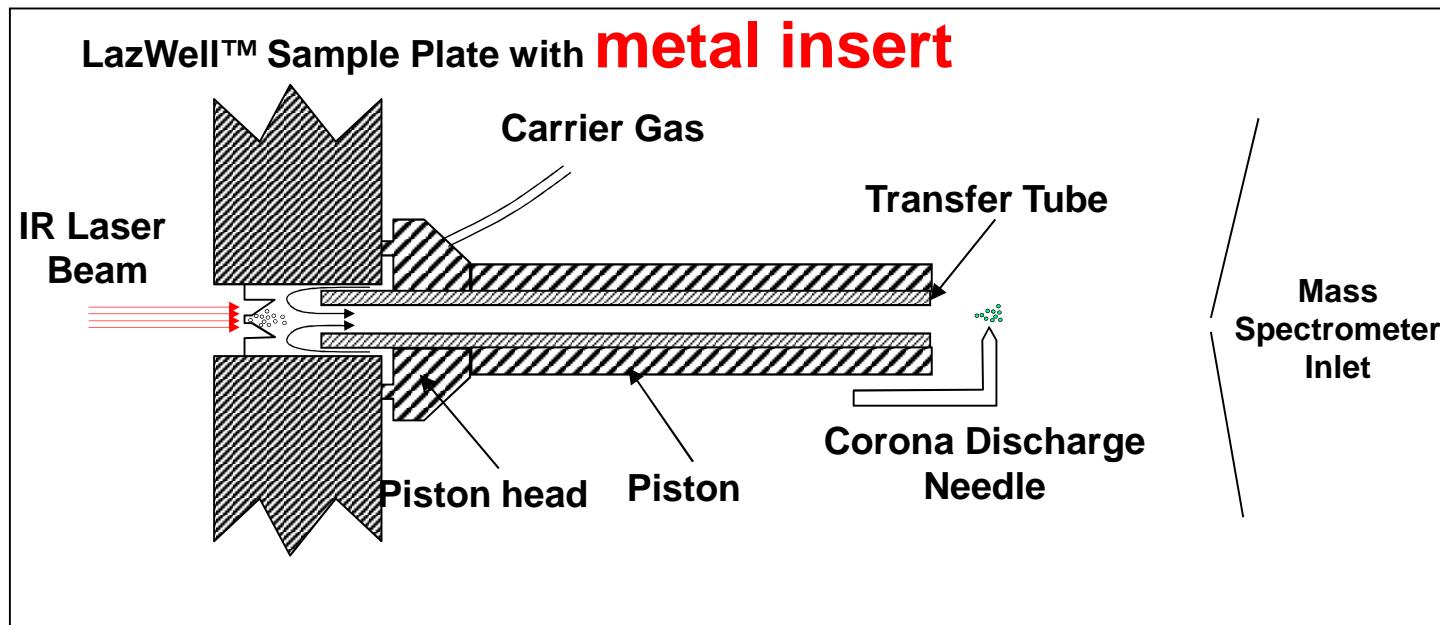
March 4th, 2014

Outline

- Fundamentals of Laser diode thermal desorption (LDTD)
- Sample Preparation Considerations
- Results and Operational Considerations
- Q & A

FUNDAMENTALS OF LASER DIODE THERMAL DESORPTION (LDTD)

What is Laser Diode Thermal Desorption (LDTD)?



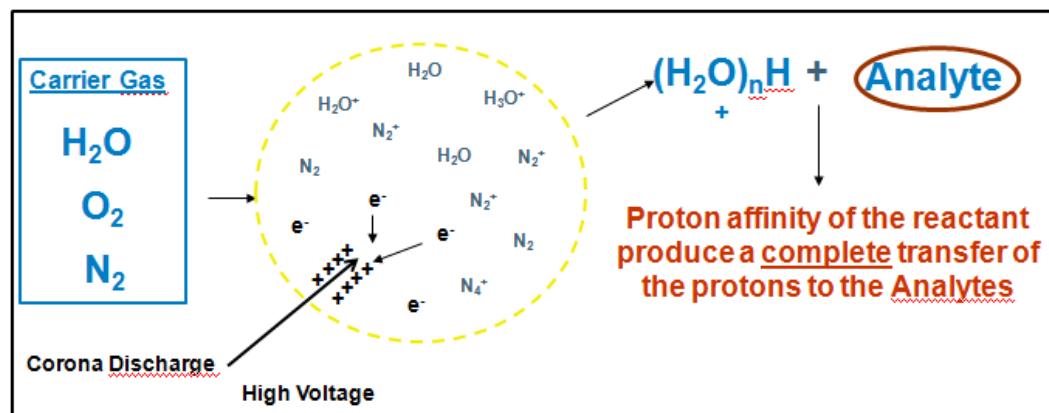
Summary of LDTD Operational Characteristics

- 1-10 µL of sample is deposited into (96/384) well plate with stainless steel base insert
- Bottom of the plate is heated by a precisely controlled, programmable infrared diode laser power gradient in seconds
- Desorption has been observed to occur at temperatures well below the bulk melting point due to weaker interaction forces at the nanoscale range¹
- Low maintenance, simple operation and change of ionization source
- No carry-over effects and less prone to matrix effects and inlet contamination than LC-ESI/APCI techniques

¹Picard, P. ; Tremblay, P; Real Paquin, E. 56th ASMS

Key differences in APCI Mechanism in LDTD vs LCMS

- APCI composition: Air with ppm level of water
- H_3O^+ (H_2O)_n cluster distribution center at n=3
- Gas temperature approximately 30 Celsius
- During typical desorption of volatiles:
 - LDTD nM/s vs LCMS mM/s
- Air 3 L/min; Corona 3 mA

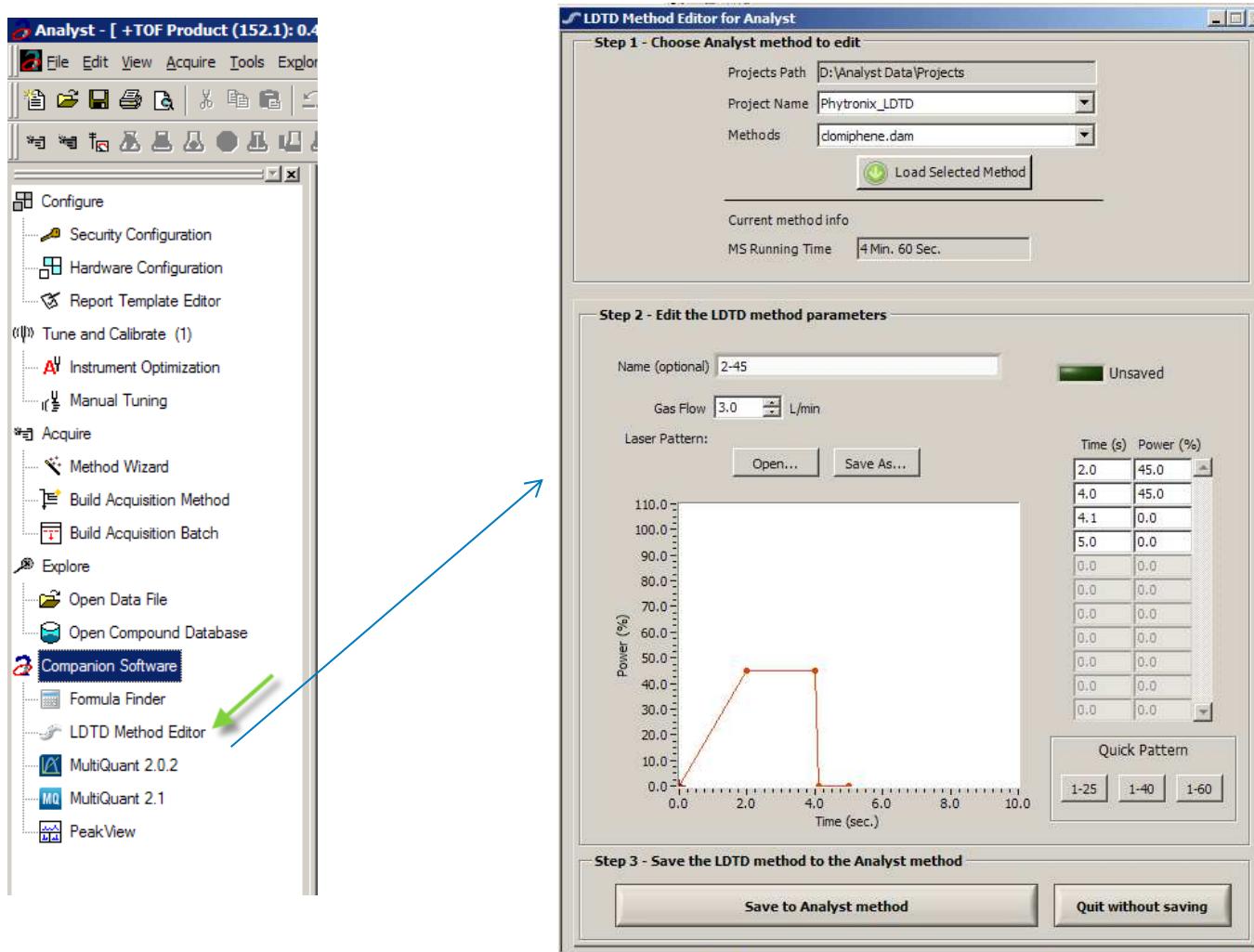


LDTD Ionization Characteristics

- A laser diode is used to heat the bottom of a metal insert 96 well plate
- Vaporized sample is carried by nitrogen gas flow to the APCI corona discharge ionization source.
- LTDI Ionization is strongly resistant to ionic suppression up to the capacity of the APCI current (2×10^{-10} M/sec)¹.
- Ionization occurring in the absence of LC mobile phase is characterized by small clusters of H_3O^+ (H_2O)_n with distribution center at n=3.
- Proton transfer to gas phase analytes under these conditions occurs with high efficiency.

¹Picard, P. ; Tremblay, P; Real Paquin, E. 56th ASMS

Embedded LDTD Power Gradient Method For Integrated Acquisition Control



LDTD Device Source Replaces Standard Ion Source

- Rapid swapping between standard ion sources and LTD
- Easily sustain LTD and UPLC workflows on the same Mass Spectrometer



Generic Laser Power Gradient

Time (s)	Power (%)	Notes
1.0	0.0	Gas Flow Stabilization
4.0	50.0	Power Ramp
6.0	50.0	Power Hold
6.1	0.0	Reduce Power
7.0	0.0	Hold at Zero
8.5-9	N/A	Travel to Next Well

The peak surface temperature is ~150 C

LDTD Sample Desorption Characteristics

- Up to 10 µL of sample is deposited into 96 well plate with stainless steel base insert.
- Desorption has been observed to occur at temperatures well below the bulk melting point of the analyte due to weaker interaction forces at the nanoscale range¹.
- LTDT has no carry-over effects and is less prone to mass spectrometer inlet contamination than LC-ESI/APCI techniques.
- Salt and other non-volatile sample components remain on the plate surface during desorption.

UPLC and LDTD Operational Cost Per Sample

UPLC	LDTD
Service Contract	\$0.09
LC Column	\$0.10
Solvent	\$0.40
Solvent Waste	\$0.02
Consumables	\$0.03
	\$0.64
Service Contract	\$0.09
96 well	\$0.50
384 well	\$0.36
	\$0.45-0.59

ROI and Initial cost and additional availability of mass spectrometry time not included in this analysis

SAMPLE PREPARATION CONSIDERATIONS

Automated ADME Assays Evaluated by LDTD

Drug-Drug Interactions	Transporters	Stability
IC50 Shift (6 / 192) Time dependent Inhibition (16 / 192) Induction	Permeability Efflux (32 / 480) Cell Uptake	Microsomal Stability (24 / 576) Tier 1 Microsomal Stability (80 / 384)
• Assay measures metabolites of specific probe substrates	• Assay measures bidirectional permeability of test compounds in multi chamber wells	• High throughput screen to measure percent metabolized in 30 minutes
• Optimized assay methodology with deuterated internal standards	• Higher Sensitivity Demands	• Generally, sets of chemically diverse compounds

Typical Sample Preparation and Analysis for ADME

- Perform assay on Tecan EVO robotic liquid handler in 96/**384** well plate
- Quench incubate with acetonitrile containing internal standard 1:2 up to 1:4 in 384 well plate*
- Centrifuge samples for 15 minutes at 3000 rpm (optional)

LC/MSMS	LDTD
<ul style="list-style-type: none">• Seal plate and submit for injection (5-25 μL) onto the LC/MSMS• Chromatography cycle time ranges from 80 to 180 seconds	<ul style="list-style-type: none">• Dilute sample if necessary• Spot 1-6 μL of sample on the LazWell™ LDTD sample plate• Dry plate under nitrogen for 6 minutes• Cycle time is under 10 seconds

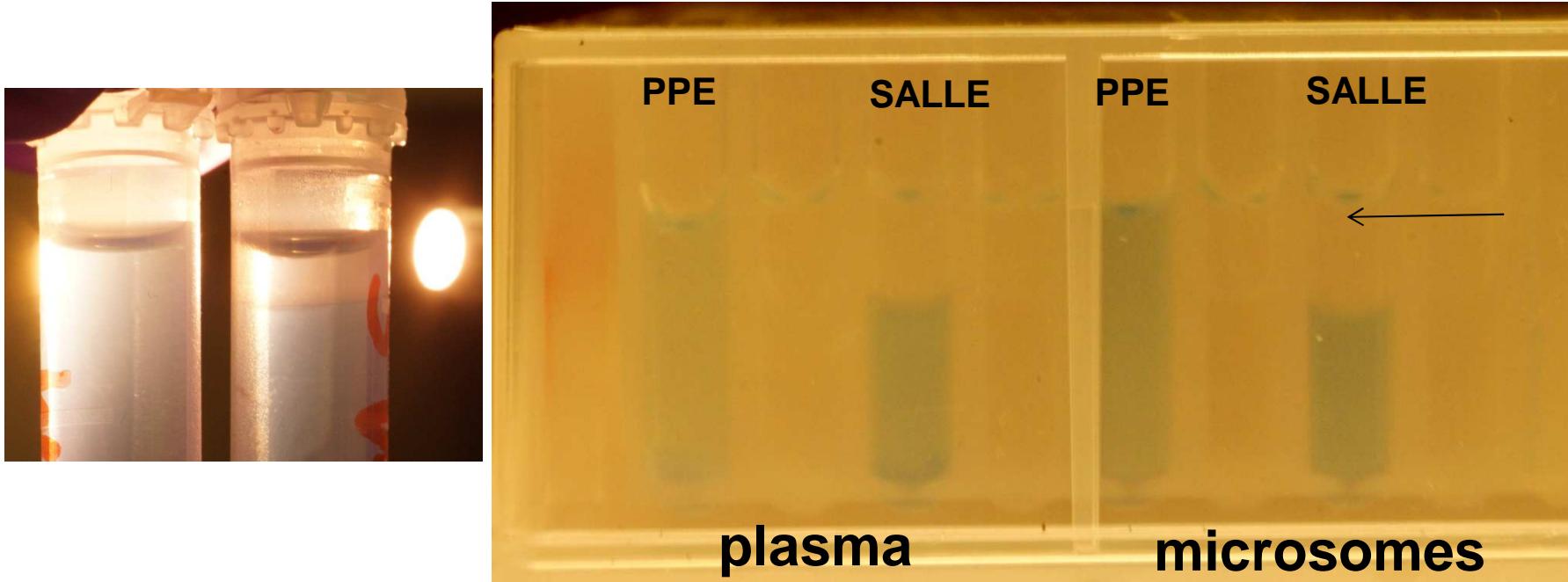
1. Salt Assisted Liquid Liquid Extraction (SALLE)
2. Dilution up to 1:19 while sustaining an aqueous component at ~ 25%

Summary of Sample Preparation Considerations

- Sample type, preparation methods and spotting volume must all be considered in order to avoid overloading the APCI Ionization Source.
- Trapping of some analytes within the non volatile sample components can contribute to signal loss.
- Sample dilution and/or more selective extraction techniques are known to increase signal 5-20x.
- Sample additives improve signal when surface or matrix interaction effects are observed
- Precise volume, placement and gentle drying

Salt-Assisted Liquid Liquid Extraction (SALLE)

- Standard acetonitrile protein precipitation method with the addition of saturated NaCl
- Spontaneous separation of immiscible top layer of acetonitrile forms in ~ 60s

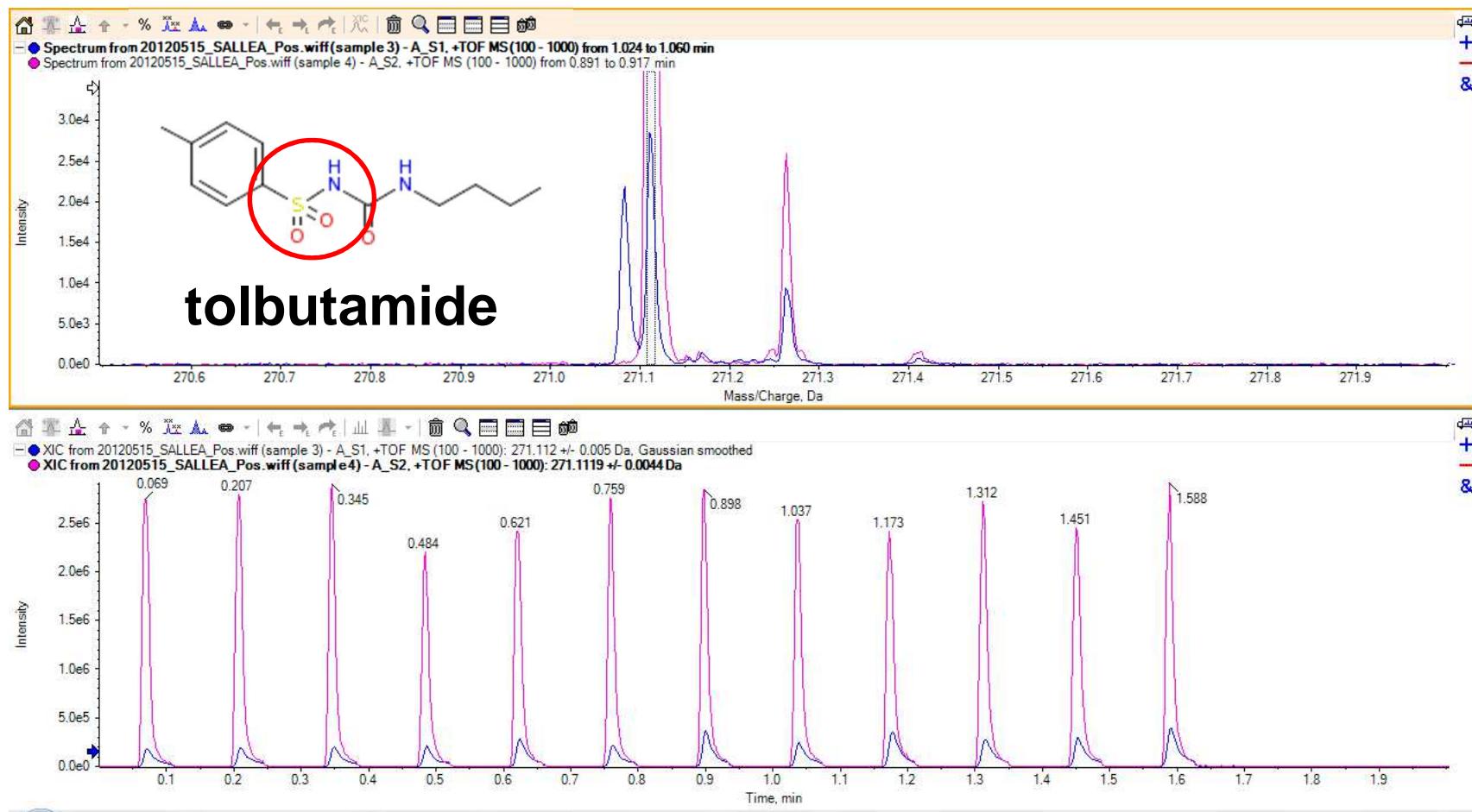


Zhang, J; Wu, H.; Kim, E; El-Shourbagy , T.A *Biomed. Chrom.* 2009, 23: 419-425

SALLE Sample Preparation for LDTD Analysis in 384 Well Plates

- Perform assay full automation protocol:
 - Prepare dosing solutions and buffers to dispense to plates
 - Incubation on Tecan EVO robotic liquid handler in 96/384 well plate
- Quench 40 µL incubate with 80 µL acetonitrile containing internal standard 1:2 384 well plate
- Add 40 µL saturated NaCl
- Wait 60s for Layer Separation
- Sample upper layer and spot 6 µL of sample on the LazWell™ LDTD sample plate
 - Specialized liquid class for spotting LDTD plates has been optimized:
 - Slow dispense (~2 µL /s) with liquid contact at the surface
 - Post-dispense delay of 1 s
- Gently dry plate under nitrogen for 6 minutes

Effect of SALLE on Response of Tolbutamide Internal Standard



Results and Operational Considerations

FDA Preferred and Acceptable In-Vitro Substrates

Table 2. Preferred and acceptable chemical substrates for in vitro experiments* (9/25/2006)

CYP	Substrate Preferred	Km (μM)	Substrate Acceptable	Km (μM)
1A2	phenacetin-O-deethylation	1.7-152	7-ethoxyresorufin-O-deethylation theophylline-N-demethylation caffeine-3-N-demethylation tacrine 1-hydroxylation	0.18-0.21 280-1230 220-1565 2.8, 16
2A6	coumarin-7-hydroxylation nicotine C-oxidation	0.30-2.3 13-162		
2B6	efavirenz hydroxylase bupropion-hydroxylation	17-23 67-168	propofol hydroxylation S-mephenytoin-N-demethylation	3.7-94 1910
2C8	Taxol 6-hydroxylation	5.4-19	amiodarone N-deethylation rosiglitazone para-hydroxylation	2.4, 4.3-7.7
2C9	tolbutamide methyl-hydroxylation S-warfarin 7-hydroxylation diclofenac 4'-hydroxylation (4 μM)	67-838 1.5-4.5 3.4-52	flurbiprofen 4'-hydroxylation phenytoin-4-hydroxylation	6-42 11.5-117
2C19	S-mephenytoin 4'-hydroxylation (20μM)	13-35	omeprazole 5-hydroxylation fluoxetine O-dealkylation	17-26 3.7-104
2D6	(±)-bufuralol 1'-hydroxylation dextromethorphan O-demethylation (4 μM)	9-15 0.44-8.5	debrisoquine 4-hydroxylation	5.6
2E1	chlorzoxazone 6-hydroxylation	39-157	p-nitrophenol 3-hydroxylation lauric acid 11-hydroxylation aniline 4-hydroxylation	3.3 130 6.3-24
3A4/5**	midazolam 1-hydroxylation testosterone 6 b -hydroxylation	1-14 52-94	erythromycin N-demethylation dextromethorphan N-demethylation triazolam 4-hydroxylation terfenadine C-hydroxylation nifedipine oxidation	33 – 88 133-710 234 15 5.1-47

* Note that this is not an exhaustive list (created May 1, 2006).

** Recommend use of 2 structurally unrelated CYP3A4/5 substrates for evaluation of in vitro CYP3A inhibition. If the drug inhibits at least one CYP3A substrate in vitro, then in vivo evaluation is warranted.

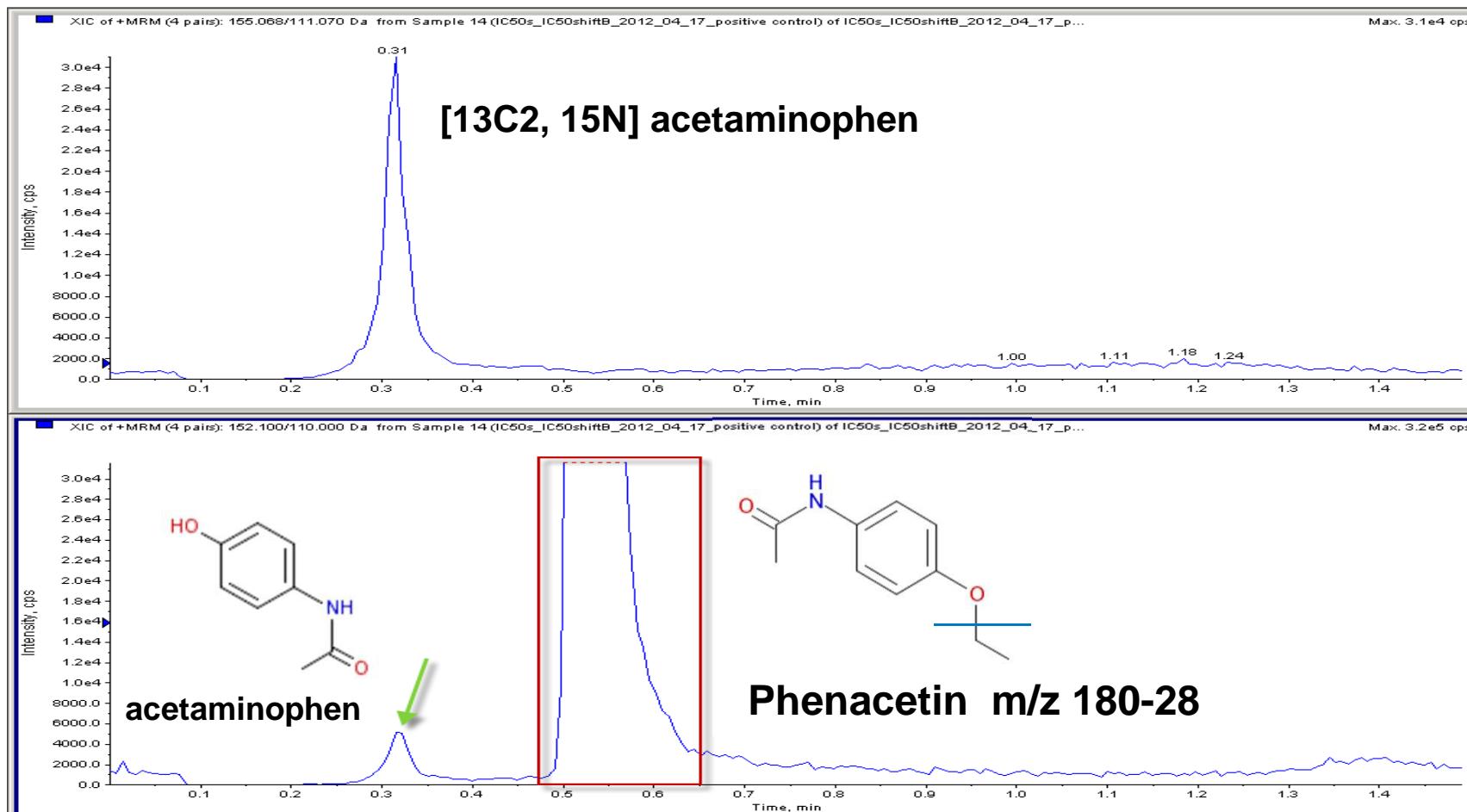
<http://www.fda.gov/downloads/drugs/developmentapprovalprocess/developmentresources/druginteractionslabeling/ucm091839.pdf>

<http://www.fda.gov/drugs/developmentapprovalprocess/developmentresources/druginteractionslabeling/ucm093664.htm>

“B”

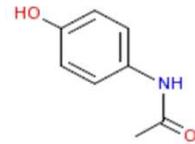
“A”

Probe Substrate Phenacetin In-source Fragmentation Interferes with Acetaminophen Positive Ionization MRM Signal

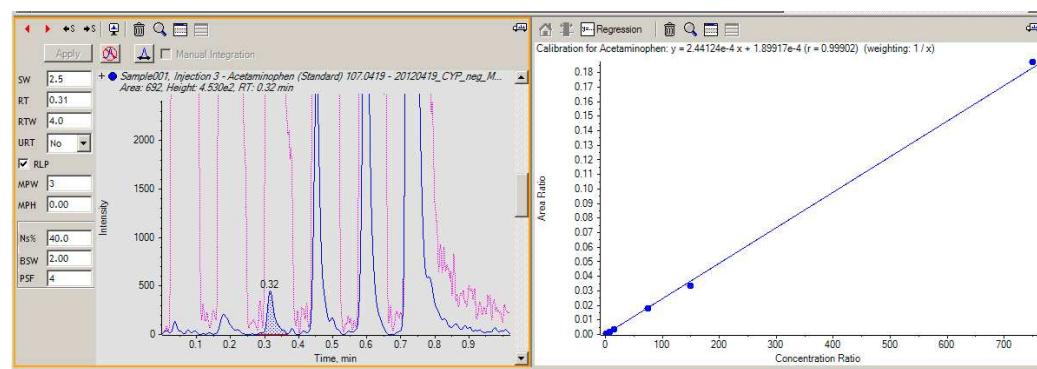


LDTD Calibration Curves 1.5-750 nM in Incubation Matrix

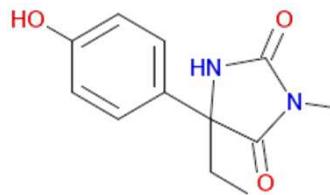
-TOF MSMS



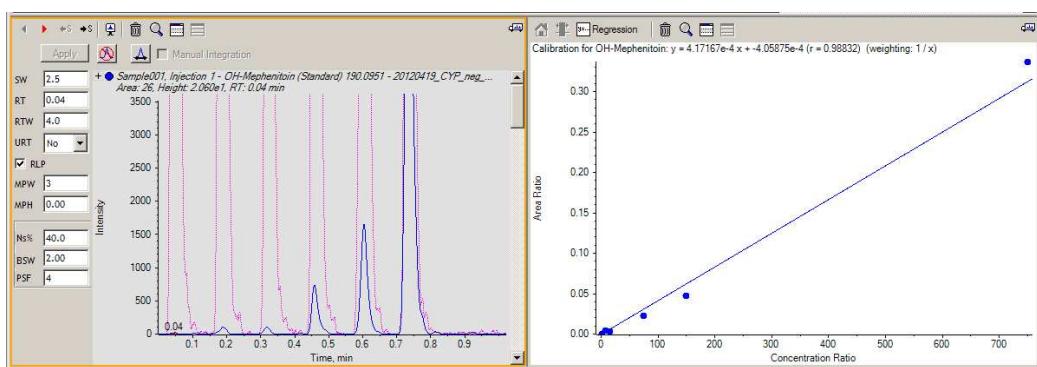
acetaminophen



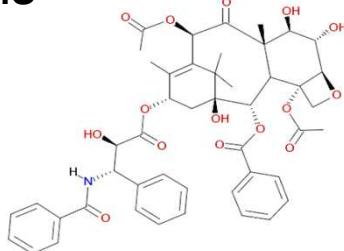
-TOF MSMS



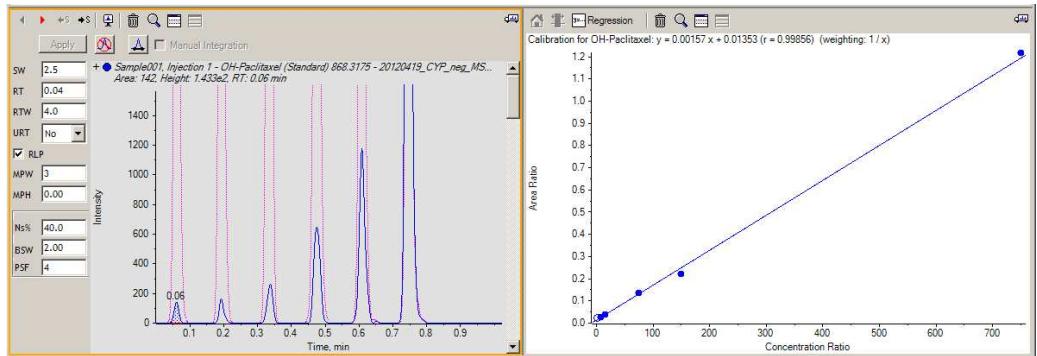
4-hydroxymephenytoin



-TOF MS



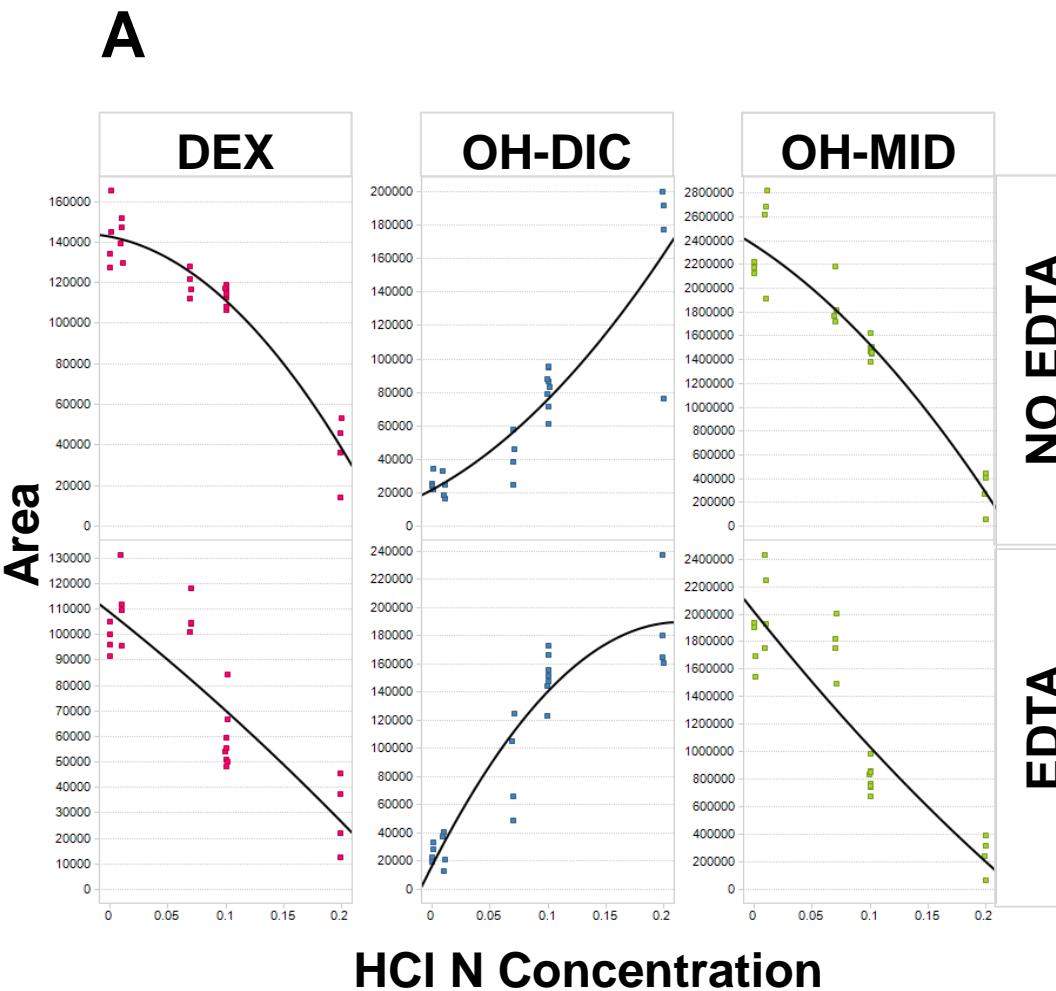
6 α -hydroxy Paclitaxel



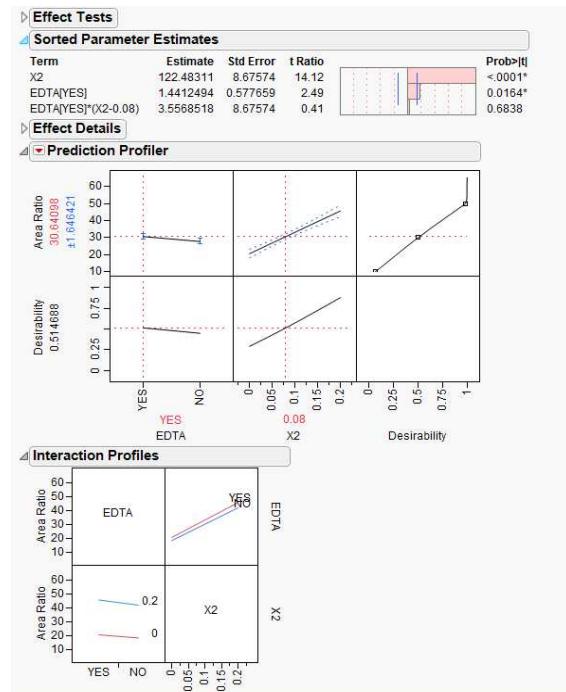
IC50 Shift Assay “A” Conditions

	CYP450 Shift Control IC50 Control	Substrate Metabolite Internal Standard
Pooled Substrates 5 min Coincubation	CYP3A4 troleandomycin ketoconazole	midazolam (2 µM) <u>1-hydroxymidazolam (OH-MID)</u> [13C3]-1-hydroxymidazolam
	CYP2D6 paroxetine quinidine	dextromethorphan (4 µM) <u>dextrorphan (DEX)</u> d3-dextrorphan
	CYP2C9 tienillic acid sulfaphenazole	diclofenac (4 µM) <u>4-hydroxydiclofenac (OH-DIC)</u> [13C6]-4-hydroxydiclofenac
	Protocol “B” CYP1A2 – phenacetin (20 µM) CYP2C8 - paclitaxel (5 µM) CYP2C19 - s-mephentyoin (20 µM)	

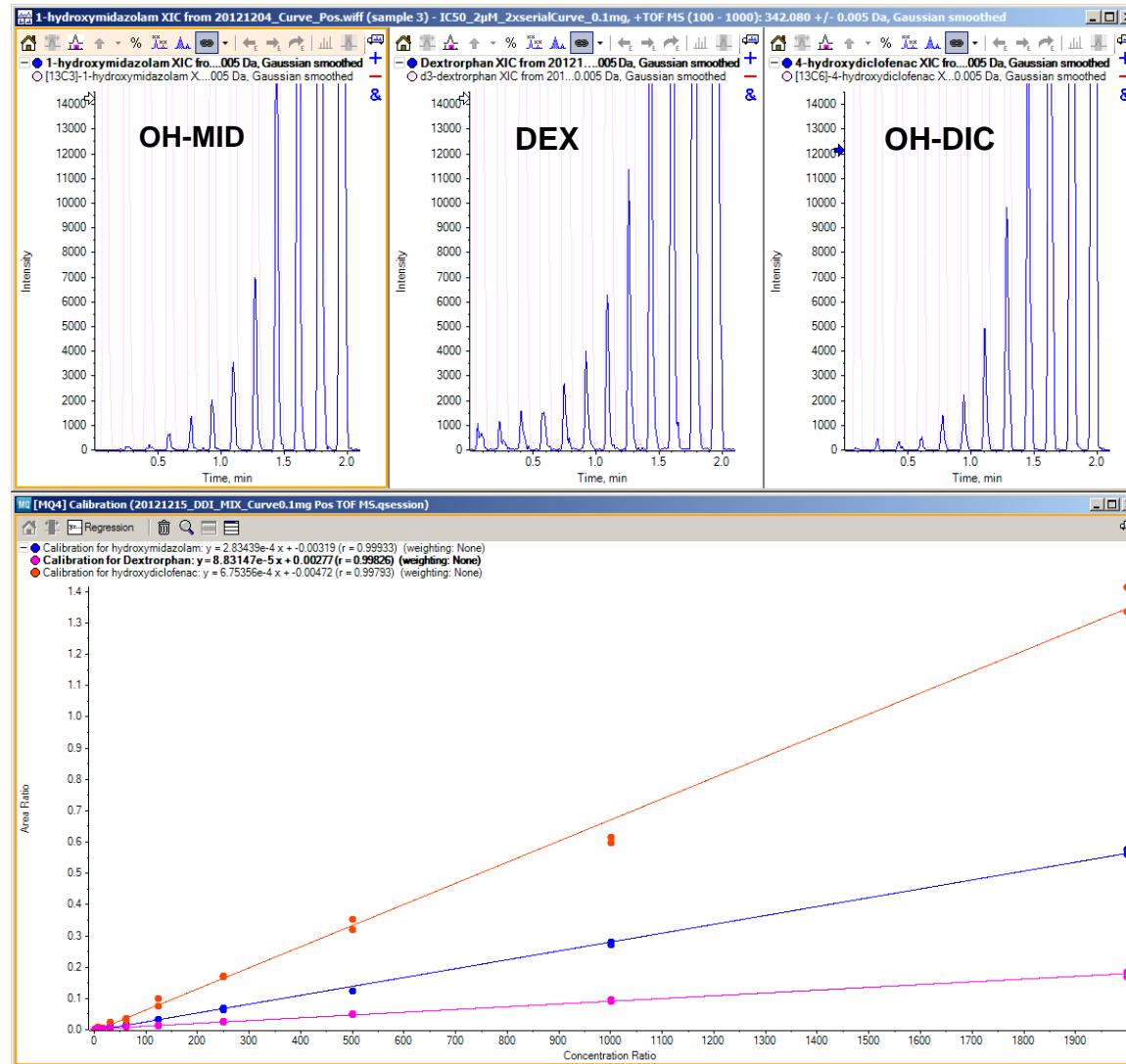
Response Curves for Metabolites with variation in HCl and EDTA



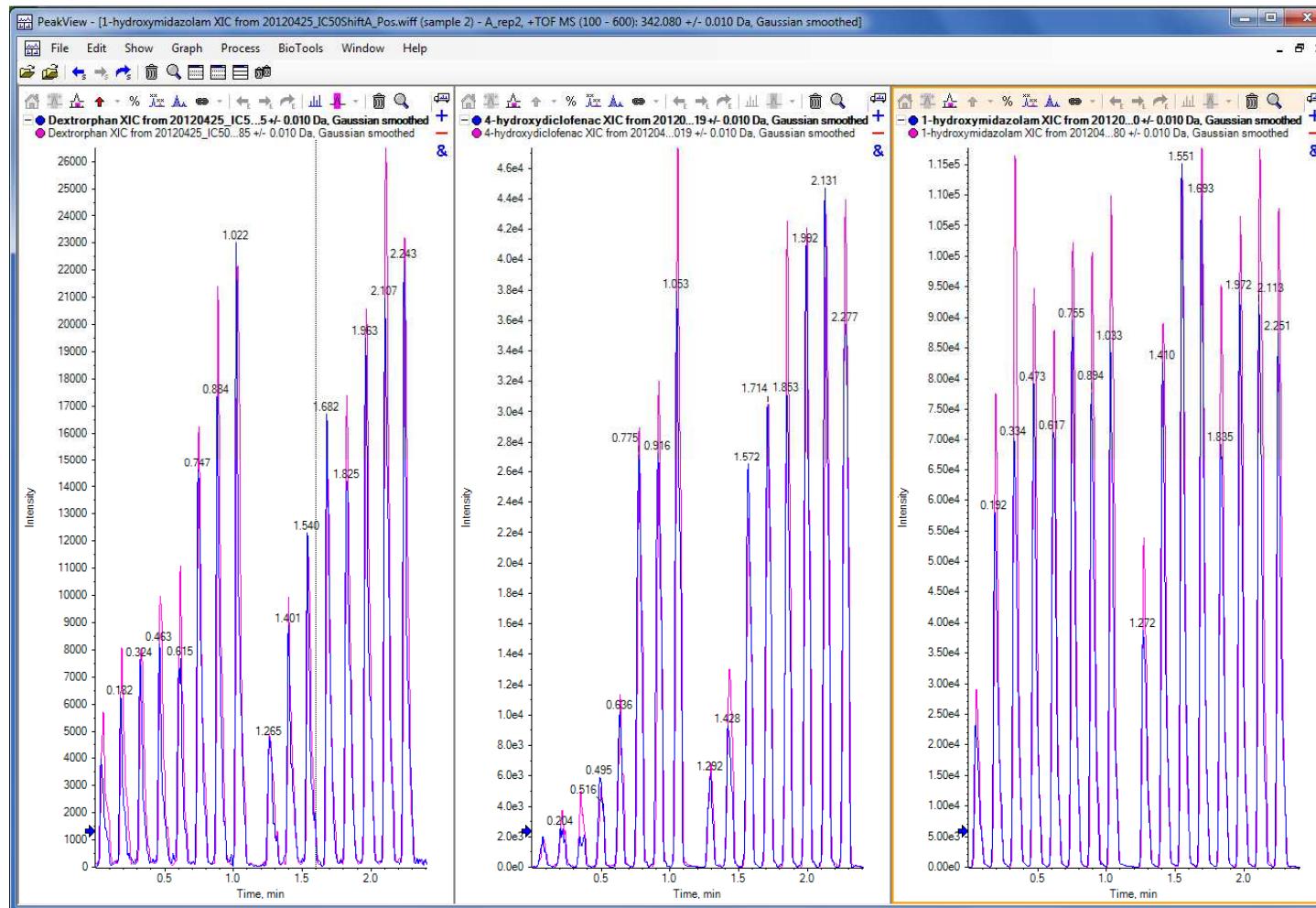
JMP™ DOE Analysis



XIC Response for Desorption of Metabolite Standards from 2-2000 nM with Calibration Curves



LDTD TOF MS Overlay of Assay Plate Duplicates for 3A4, 2D6, and 2C9



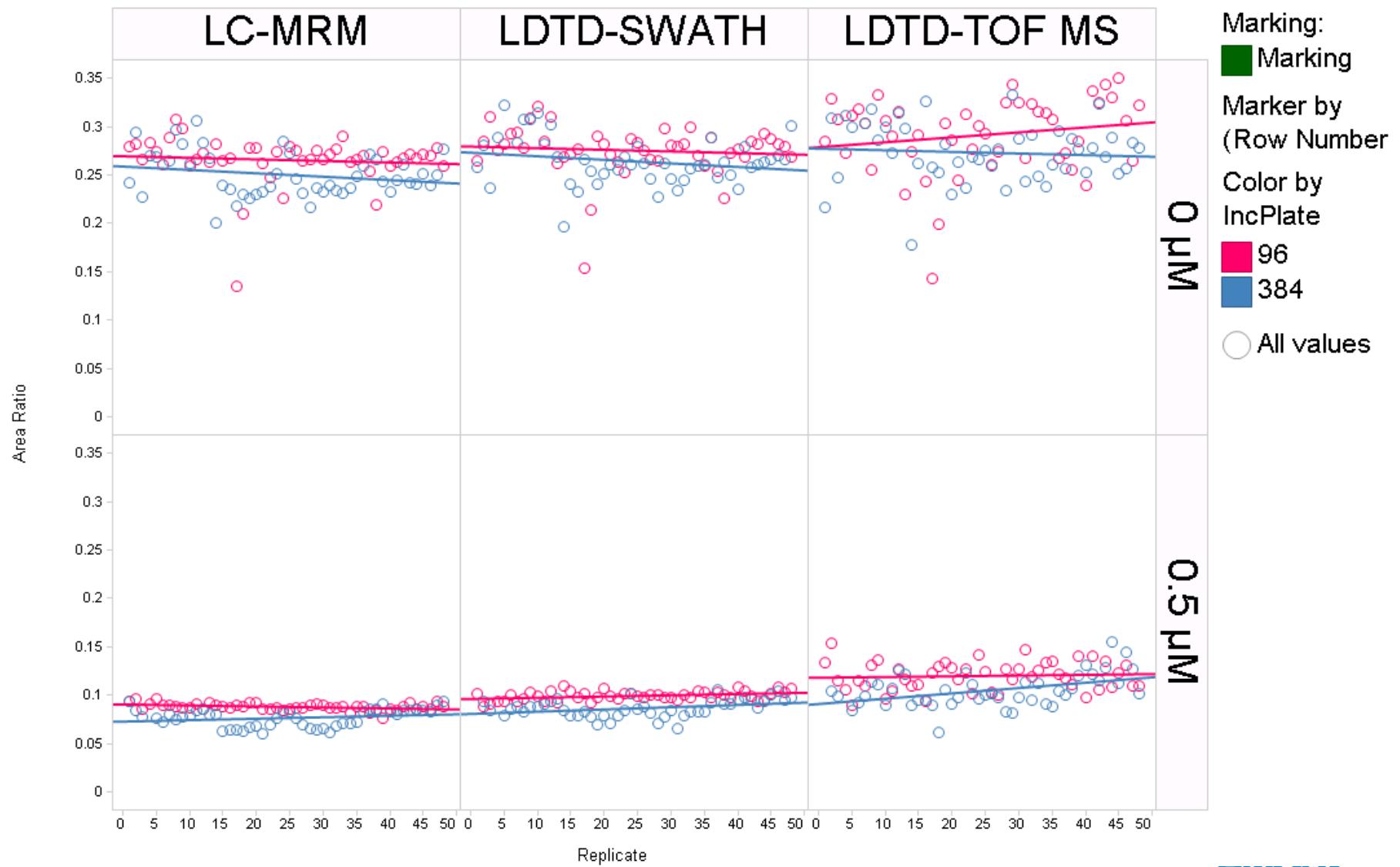
LC-MRM vs LDTD-TOF for IC50 Shift

Probe	LCMSMS	LDTD	IC50 (SE) / Shift
Phenacetin	<p>aminobenzotriazole, phenacetin (20μM), acetaminophen</p>	<p>LDTD, control, phenacetin (20μM), acetaminophen</p>	LCMSMS 12.1 (1.63) / 20.7 LDTD 10.7 (1.20) / 23.3
Diclophenac	<p>tenoxic acid, Diclofenac (4μM), 4-hydroxydiclofenac</p>	<p>tenoxic acid, Diclofenac (4μM), 4-hydroxydiclofenac</p>	LCMSMS 0.089 (0.02) / 23.7 LDTD 0.0876 (0.01) / 17.5
Midazolam	<p>troleanomycin, Midazolam (2μM), 1-hydroxymidazolam</p>	<p>troleanomycin, Midazolam (2μM), 1-hydroxymidazolam</p>	LCMSMS 2.43 (0.30) / 3.48 LDTD 2.89 (0.27) / 2.49

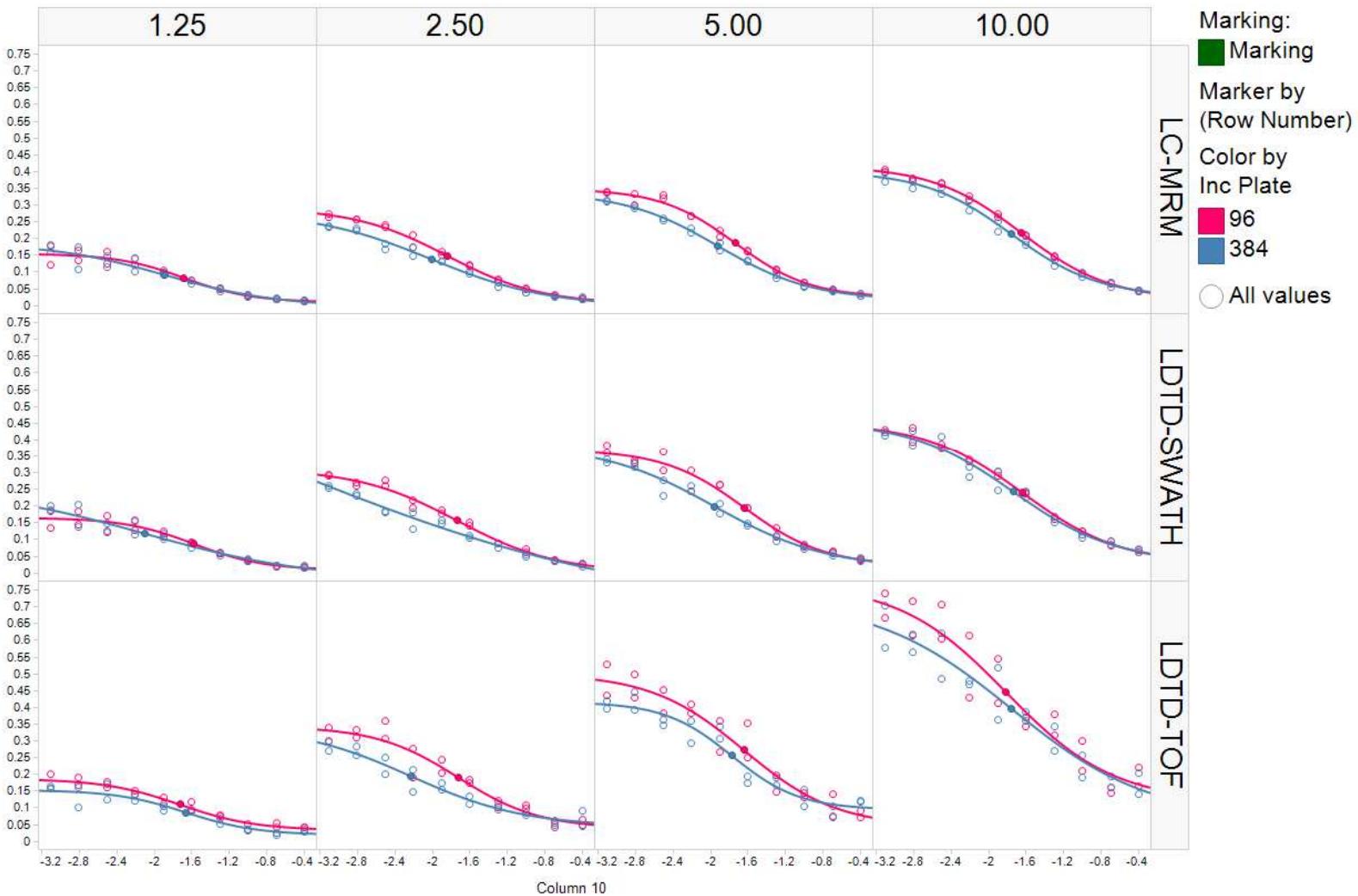
Comparison Study K_i incubation in 96 vs 384 plates

- **Substrate** Midazolam Conc. (μM):
 - 1.25, 2.5, 5 & 10 ($K_m = 2.5$)
- **Inhibitor** Ketoconazole Conc. (μM):
 - 0.0078, 0.0156, 0.0312, 0.0624, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4
- HLM (mg/mL): 0.1
- NADPH (mM): 1
- Incubation Time (min): 5
- Incubation volume (μL): 300 (96) / 100 (384)
- Quench samples / split extract and run with LC-MRM / LDTD TOF MS / LDTD SWATH

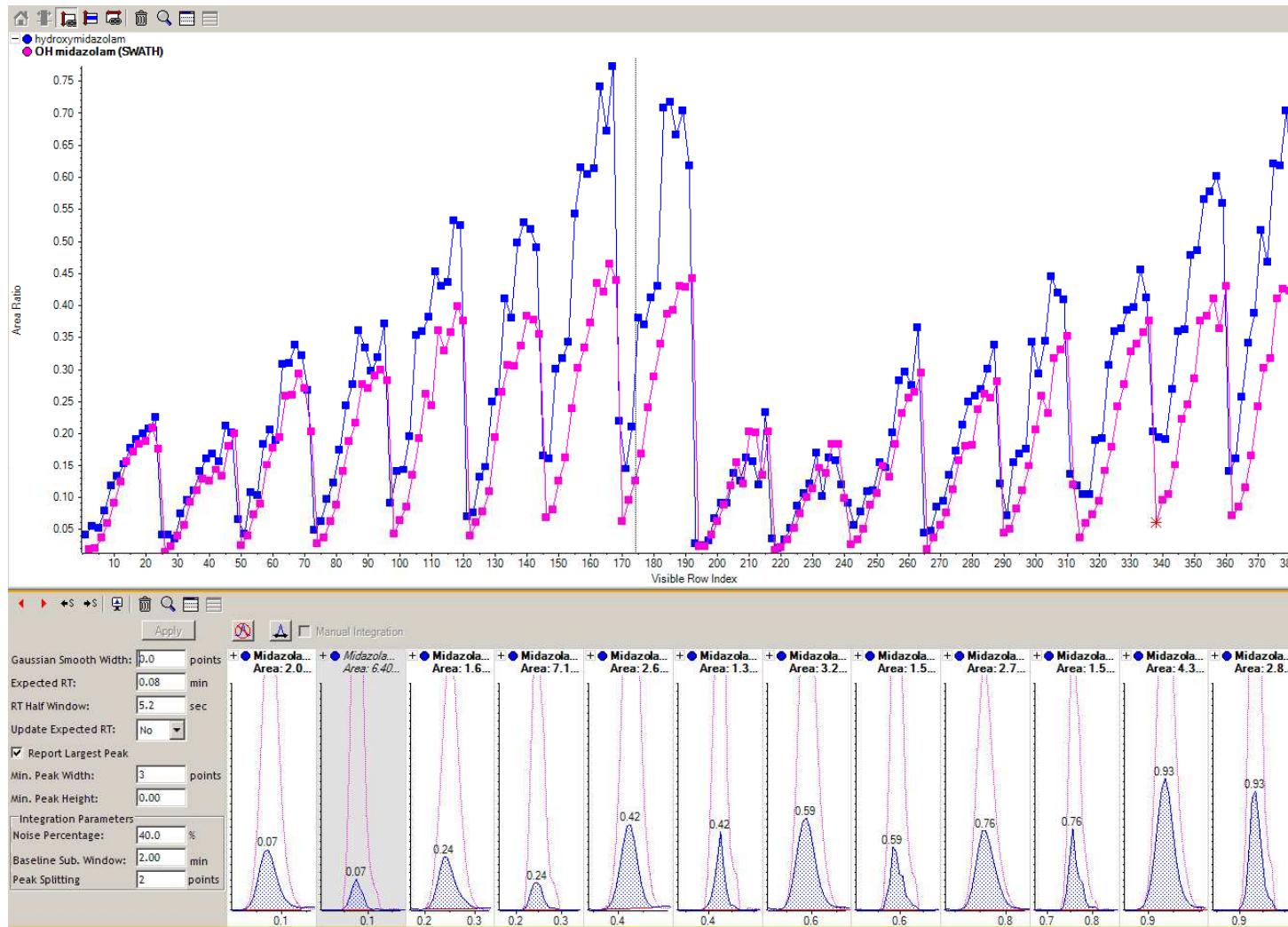
Scatter Plot



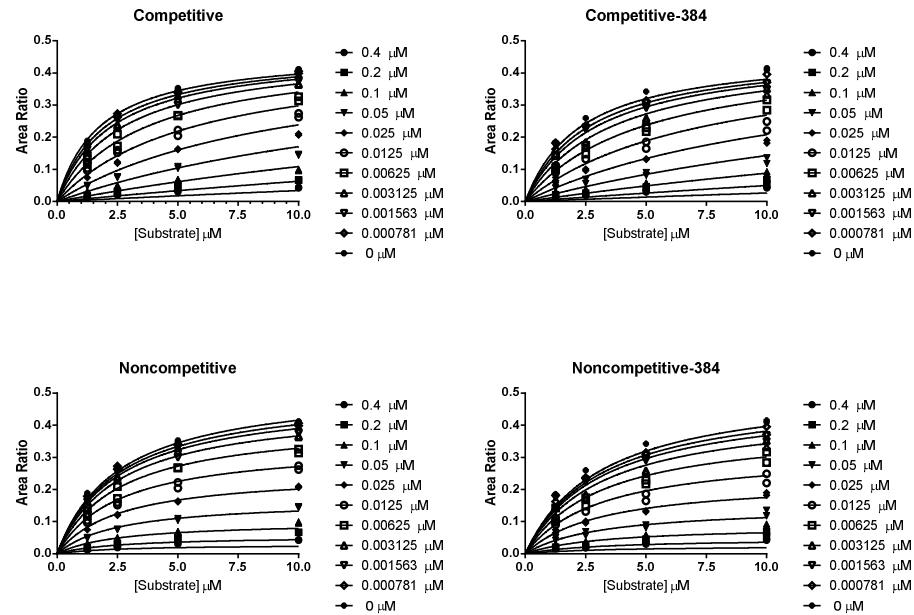
Ki Comparison 96 and 384 WP Incubation



Metric Plot Midazolam K_i Study with LDTD TOF MS and SWATH

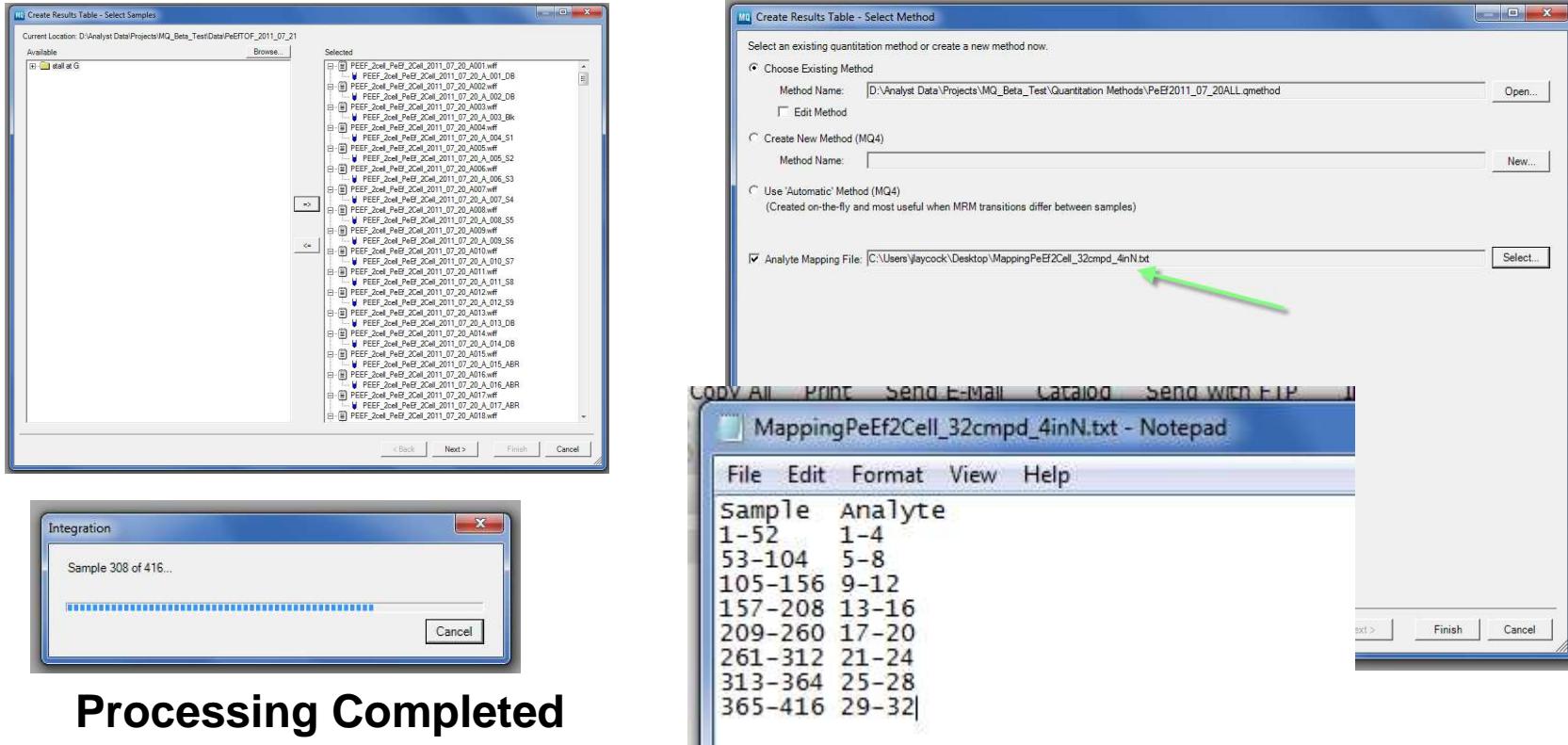


Ki Results



Inc. Plate	Competitive	Noncompetitive	Uncompetitive	Mixed Model
96 LC-MRM	0.005675	0.02351	0.01449	0.01330
384 LTD-SWATH	0.0057	0.01978	0.01119	0.01523

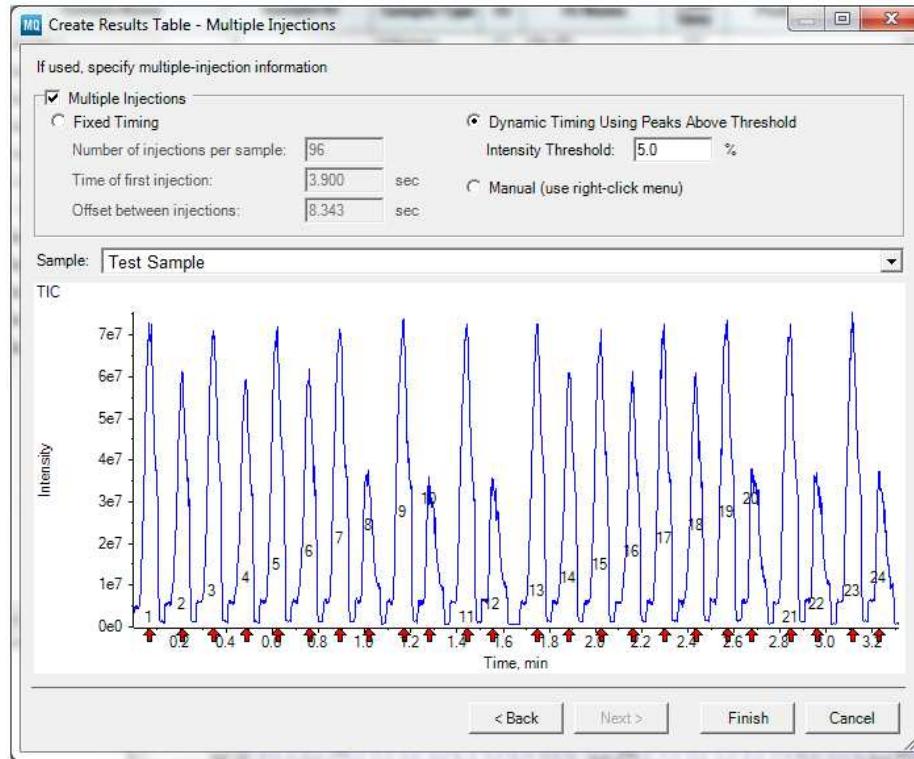
Mapping of Extraction Masses to Select Ranges of Samples Within the Selected Data



**Processing Completed
in ~1 minute**

Process multiple samples together, specifying the union of their analytes, rather than creating a separate Results Table for each analyte.

Enhanced Processing of Multiple Injection Data (Beta software version)



Ability to manually adjust/add/remove peak location and save to a custom timing file

Acknowledgements

- Yihong Zhou
- Raju Subramanian
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- Lyle Burton, ABSciex