

Efficient Multidisciplinary NMR and MS Structure Elucidation in the Age of High Sensitivity NMR

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Novatia

■ Business Need

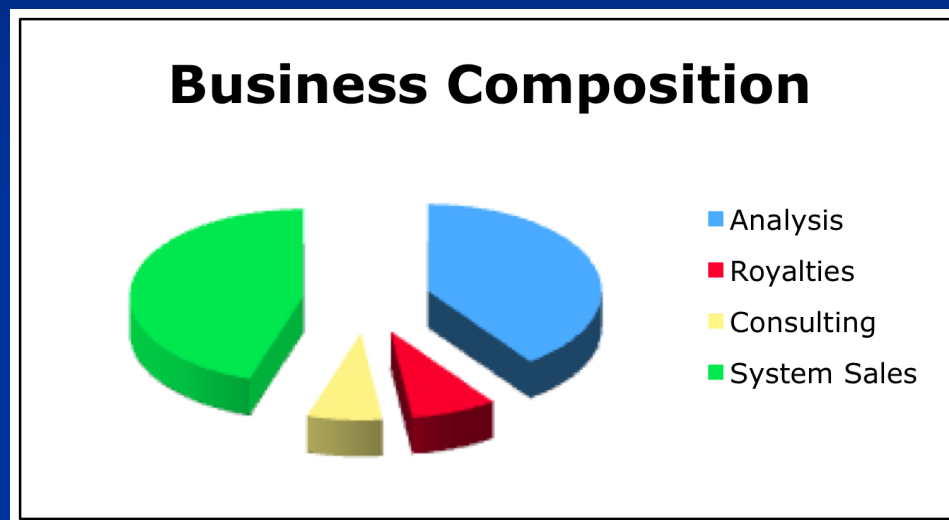
- Analytical Applications Gap
 - Instrument Vendor
 - Academic Researcher
 - End User

■ Capabilities

- 5 Scientists
- 3000 sq ft lab & office space
- 4 LCMS and 1 500 MHz NMR

■ Expertise

- HT Analysis (OligoHTCS and OMNMR)
- Software Development (ESI MS decon, metabolite ID)
- Trace Mixture Analysis (integrated MS & NMR)
- Biomolecule Analysis (NMR & MS)



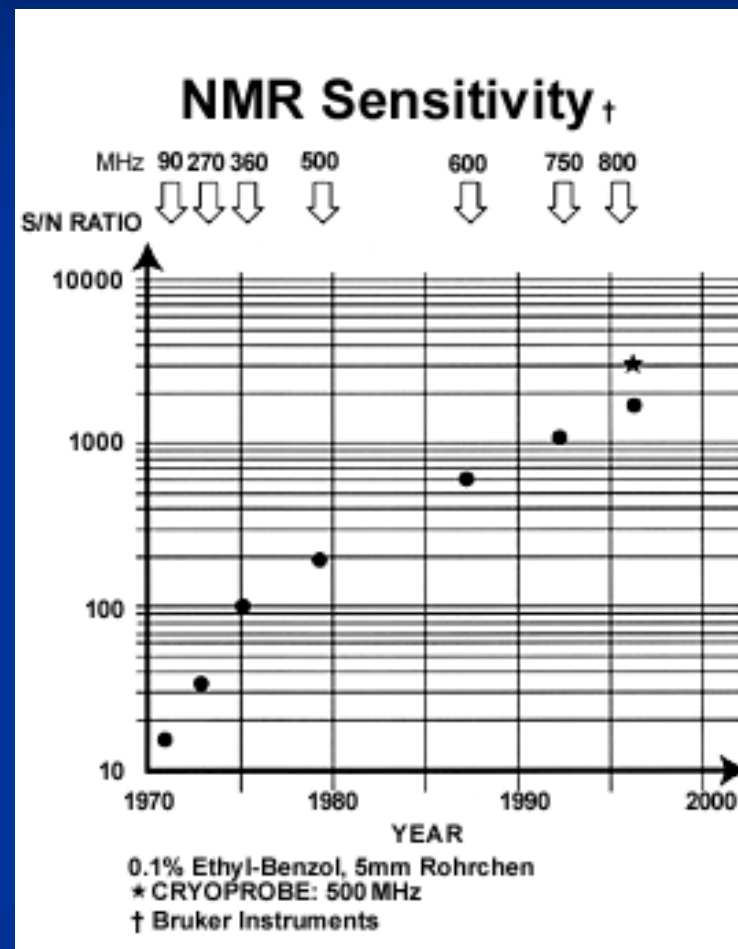
MS & NMR Structure Elucidation

- Past Isolation and Elucidation Projects
 - Medical Devices
 - Patches & Lotions
 - Cosmetics
 - Pharmaceuticals
 - Natural Products
- Challenges for NMR in MS/NMR Project
 - *Low Sensitivity (dilution factor)*
 - *Sample Purity (pollution factor)*

NMR Sensitivity

- Sensitivity Gains
 - Increasing Field Strength
 - \$\$\$
 - Better Probe Electronics
 - incremental
 - Dynamic Nuclear Polarization
 - routinely applicable?

- High Sensitivity Probes
 - 3 mm / 1.7 mm tube
 - cryo / cold Probes
 - microcoil probe



High Sensitivity NMR

- Protasis / MRM CapNMR
 - High mass sensitivity
 - Extreme trace superiority
 - Facility friendly
 - Low cost



CapNMR Sensitivity Comparison

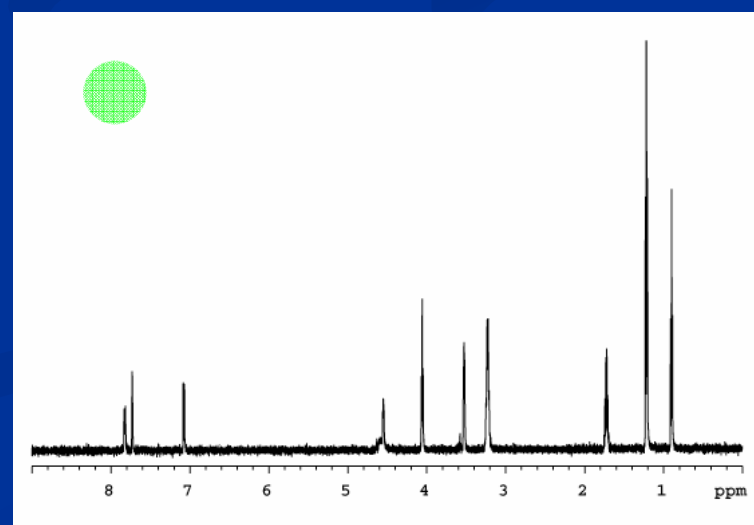
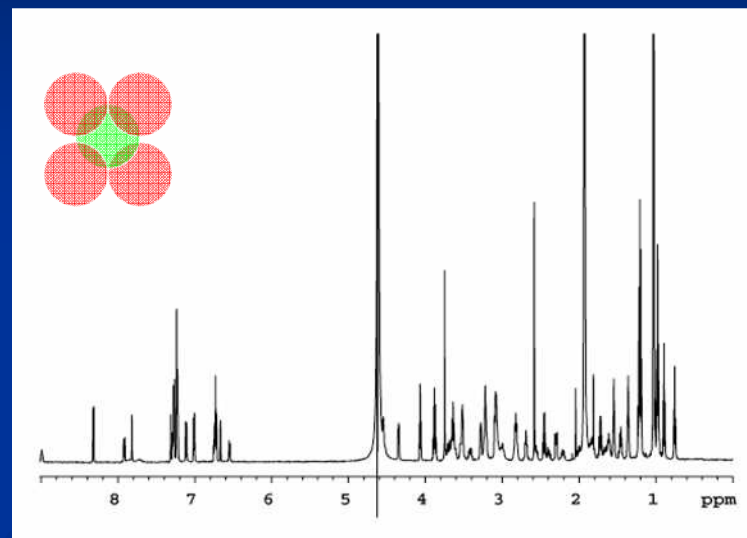
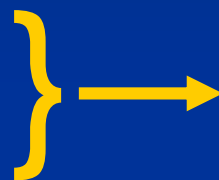
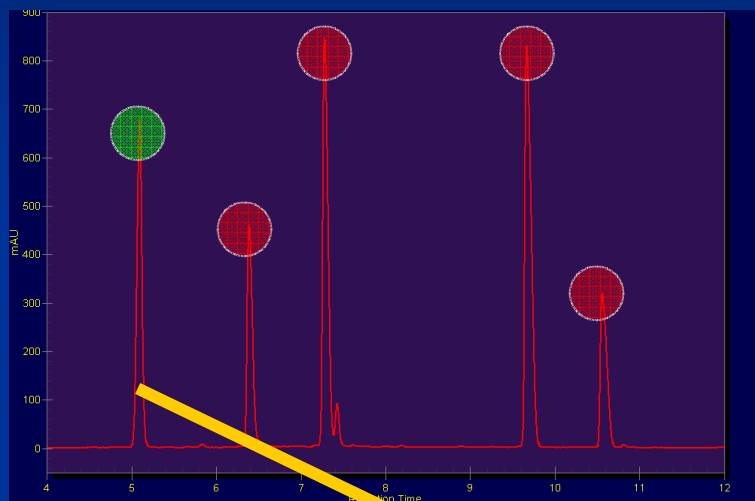
Table 2. Comparison of Relative Mass Sensitivity and Data Acquisition Times Normalized to a Fixed Mass in the Observe Volume of Different Types of 600-MHz NMR Probes^a

relative figure of merit	conventional 5 mm	conventional LC flow	specialty trace	1 mm ^b	cold ^c and CapNMR
S/N	1	1.3	2	4	10
acquisition time	100	60	25	6.3	1

^a Mass sensitivity is defined in eq 2, and all S/N measurements were made on the anomeric proton of sucrose at 5.4 ppm in D₂O; single scan. Data for the conventional-scale LC flow probe and specialty trace probe were derived from promotional data from the manufacturer; line broadening unspecified. Data for the conventional 5-mm probe, the 1-mm probe, and the CapNMR probe were processed using line broadening of 0.7 Hz. Spectra for the conventional 5-mm probe and CapNMR probe were acquired in our laboratory on a Varian Inova 600 MHz spectrometer. Data for the cold probes were acquired using line broadening of 1.0, and are not tabled separately; see text. Relative data acquisition times were computed using eq 6 for a fixed mass (where n is proportional to acquisition time). The tables serve only as a guide, since exact relationships cannot be computed from nonidentical data acquisition conditions. ^b See ref 25. ^c See refs 5, 20.

Olson, D.L., Norcross, J.A., O'Neil-Johnson, M., Molitor, P.R., Detlefsen, D.J., Wilson, A.G., and Peck, T.L. "Microflow NMR: Concepts and Capabilities" Analytical Chemistry 76(10) pp 2966-74, 2004.

NMR & Mixtures Don't Mix



What about LC-NMR?

- The Good
 - Well developed vendor solutions
 - Body of expertise
- The Bad
 - Never on flow because of low sensitivity
 - Peak volume matching limits sensitivity
- The Growing Consensus
 - ... for the occasional identification of impurities, a *decoupled, preparative approach* may sometimes offer the best combination of efficiency, sensitivity and flexibility (Sharman and Jones, *Magn. Reson. Chem.* 2003, 448-454).

Where does that leave Us?

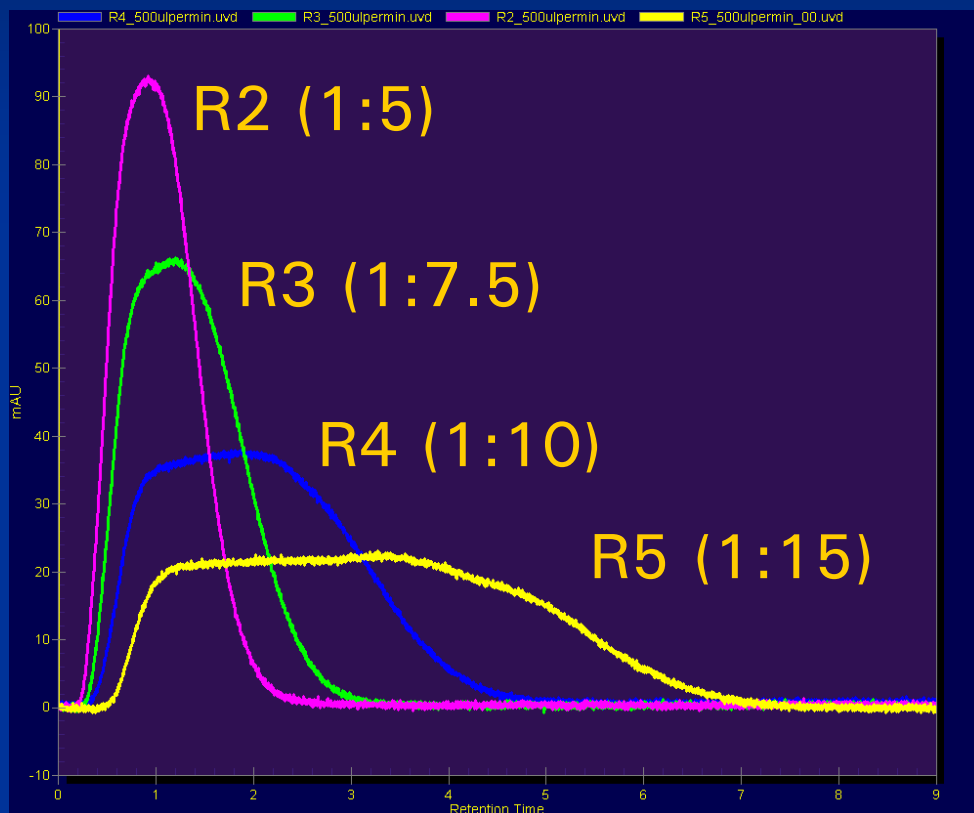
- NMR should be employed in elucidation ...
 - after exhaustive MS analysis
 - with high sensitivity NMR probes
 - using offline sample preparation...
 - leveraging chromatographic expertise
 - in a fast, robust and routine manner

SepNMR Overview



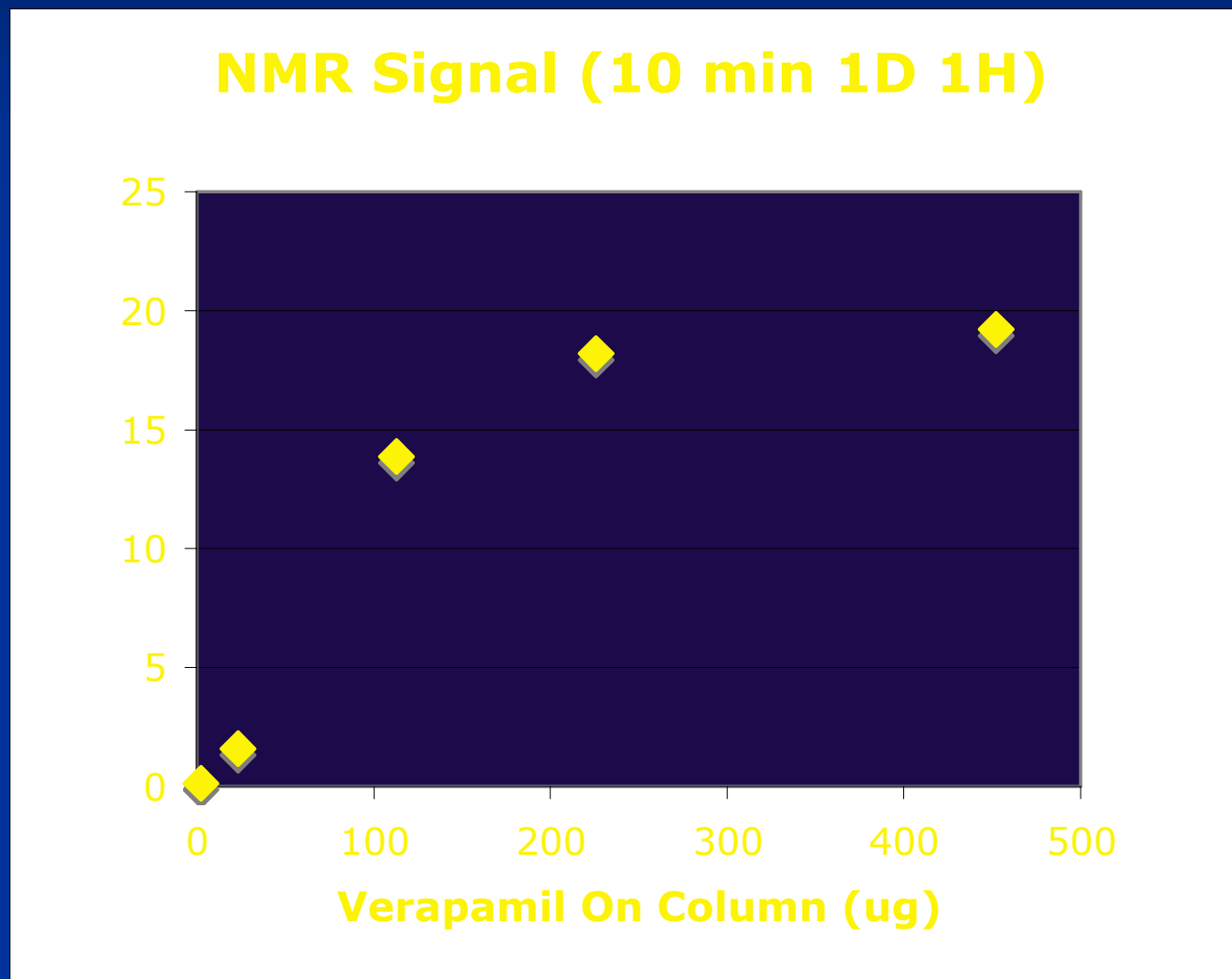
- Integrated System for Trace NMR Sample Preparation
- Modified 2D Chromatography
- Michrom Paradigm MS4
 - 4 pump system
 - 100 ul/min to 5 ml/min
 - 2-10 port valves
 - TTL Input / Output
 - UV-Vis w / nanoflow cell
- Leap Pal Autosampler
- Custom Methods and Software

Mixing Profile

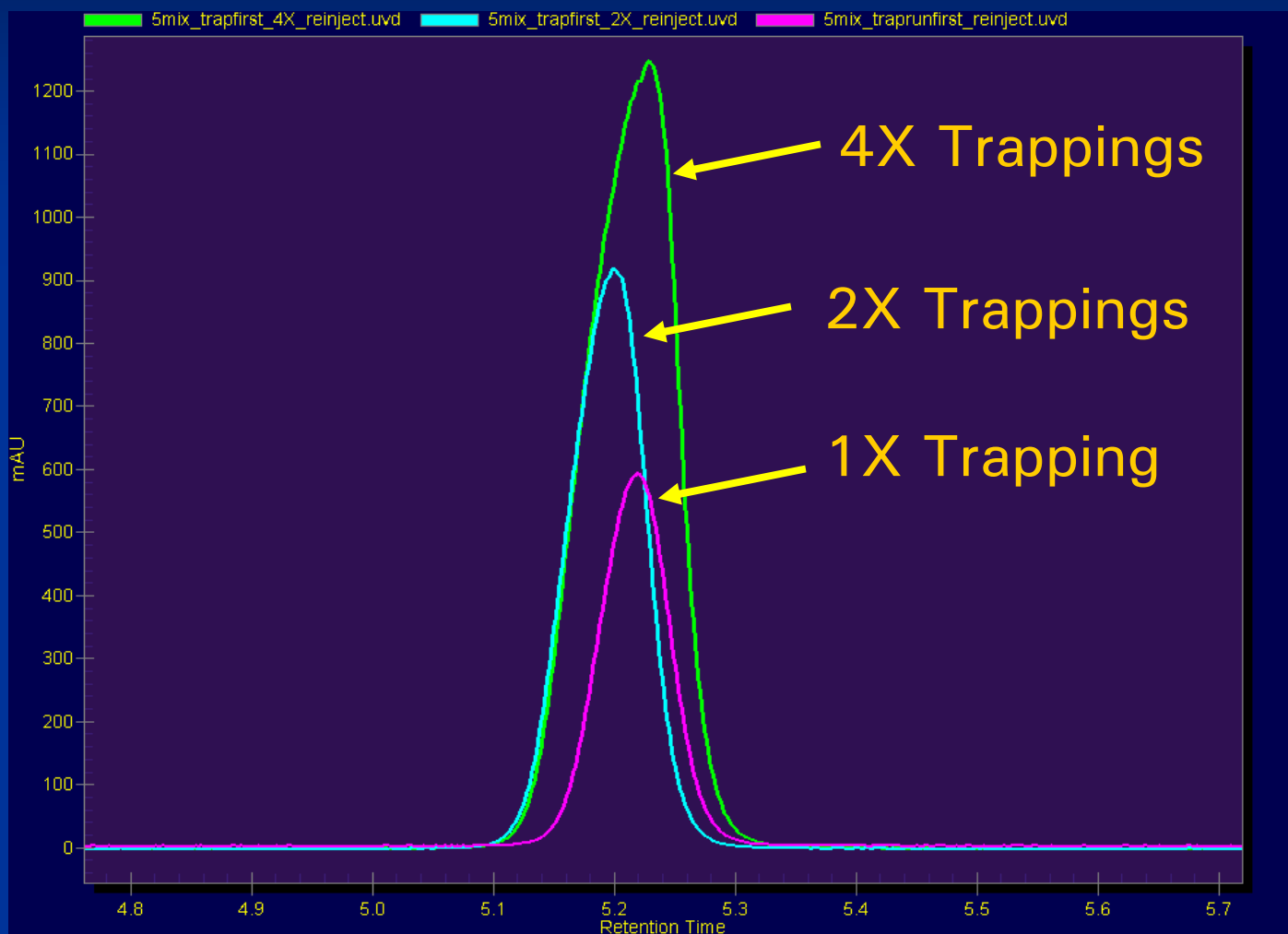


- Controlled Dilution & Mixing
 - Increase aqueous content of peak to facilitate sample recapture
 - 5 computer controlled split-flow settings allow for variable dilution
 - Dilution factor independent of initial HPLC separation

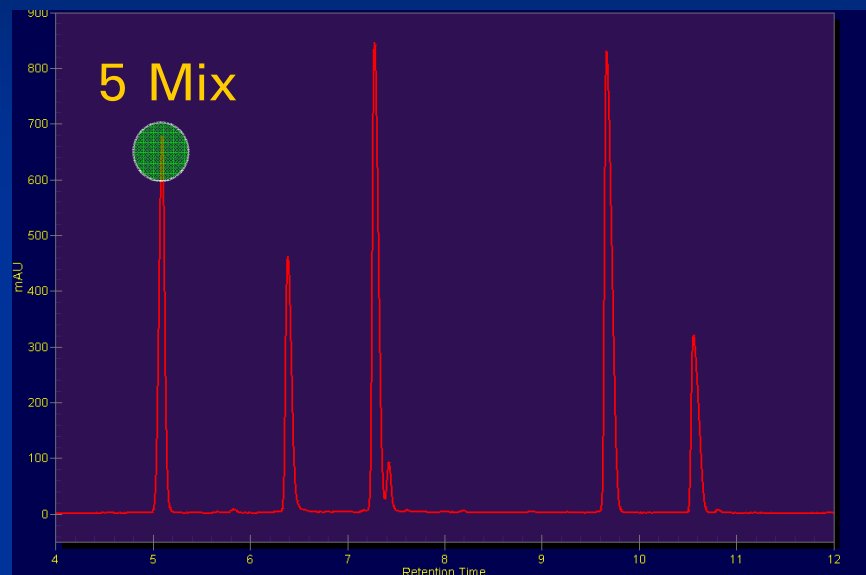
Column Trapping Capacity



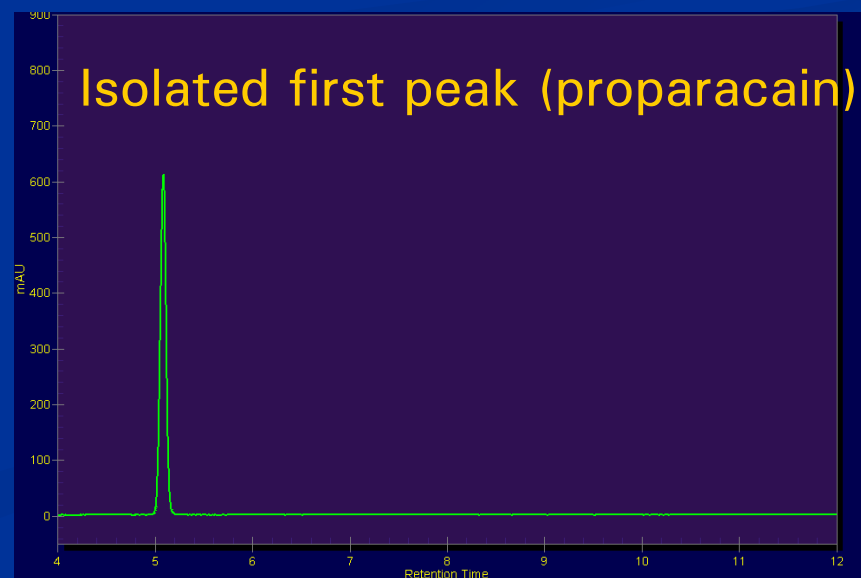
Single Peak Accumulation



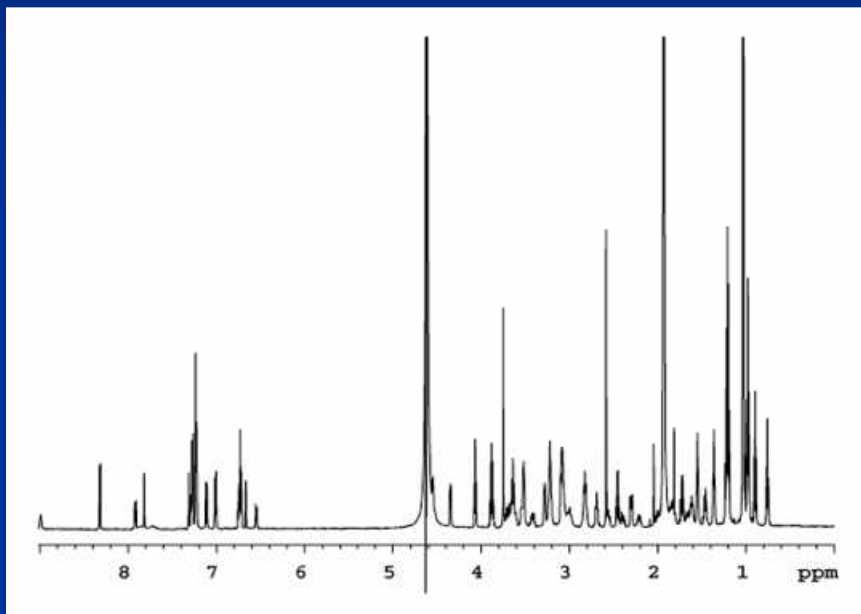
Model Compound System



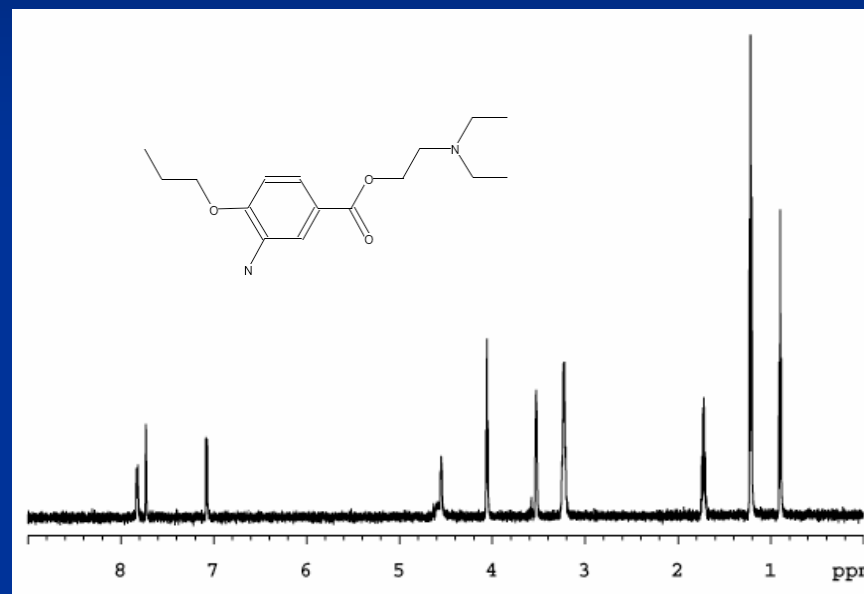
Sample:
5 ug / ul of 5 model compounds
Inject 50 ul on column
First peak elutes @ 20% organic



Isolated Model Compound NMR

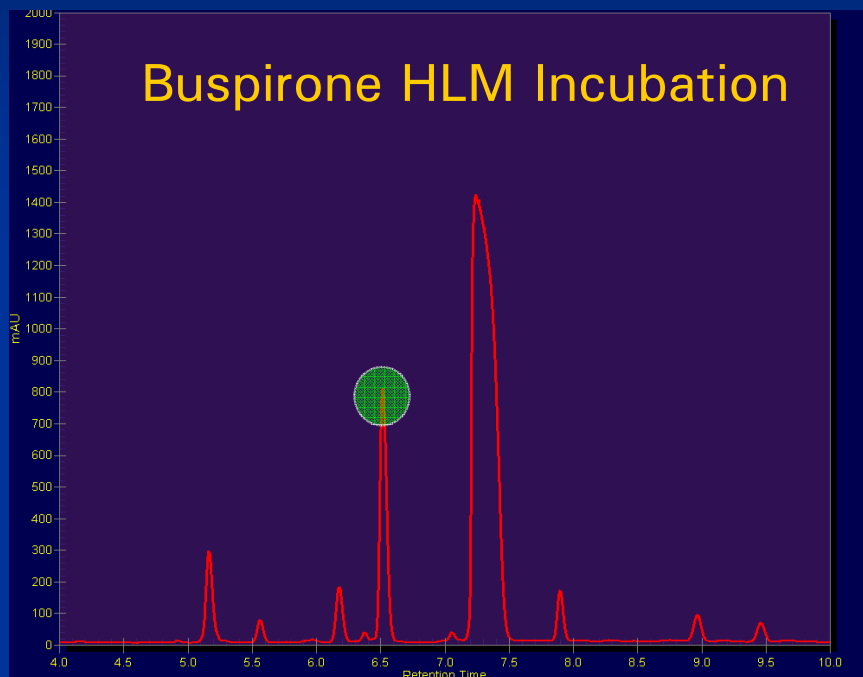


- 5 Model Compound Mixture
 - CapNMR at 600 MHz
 - 64 Scans (4 minutes)
 - 5 ug of 5 components



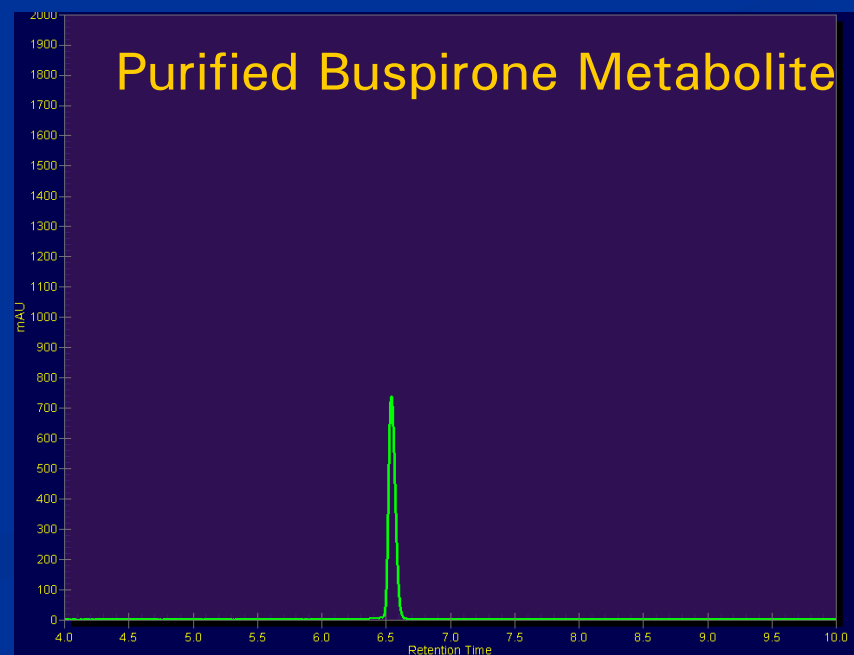
- Isolated proparacain
 - CapNMR at 600 MHz
 - 16 Scans (1 minute)
 - 5 ug of 1 component on column

Buspirone Metabolite Elucidation



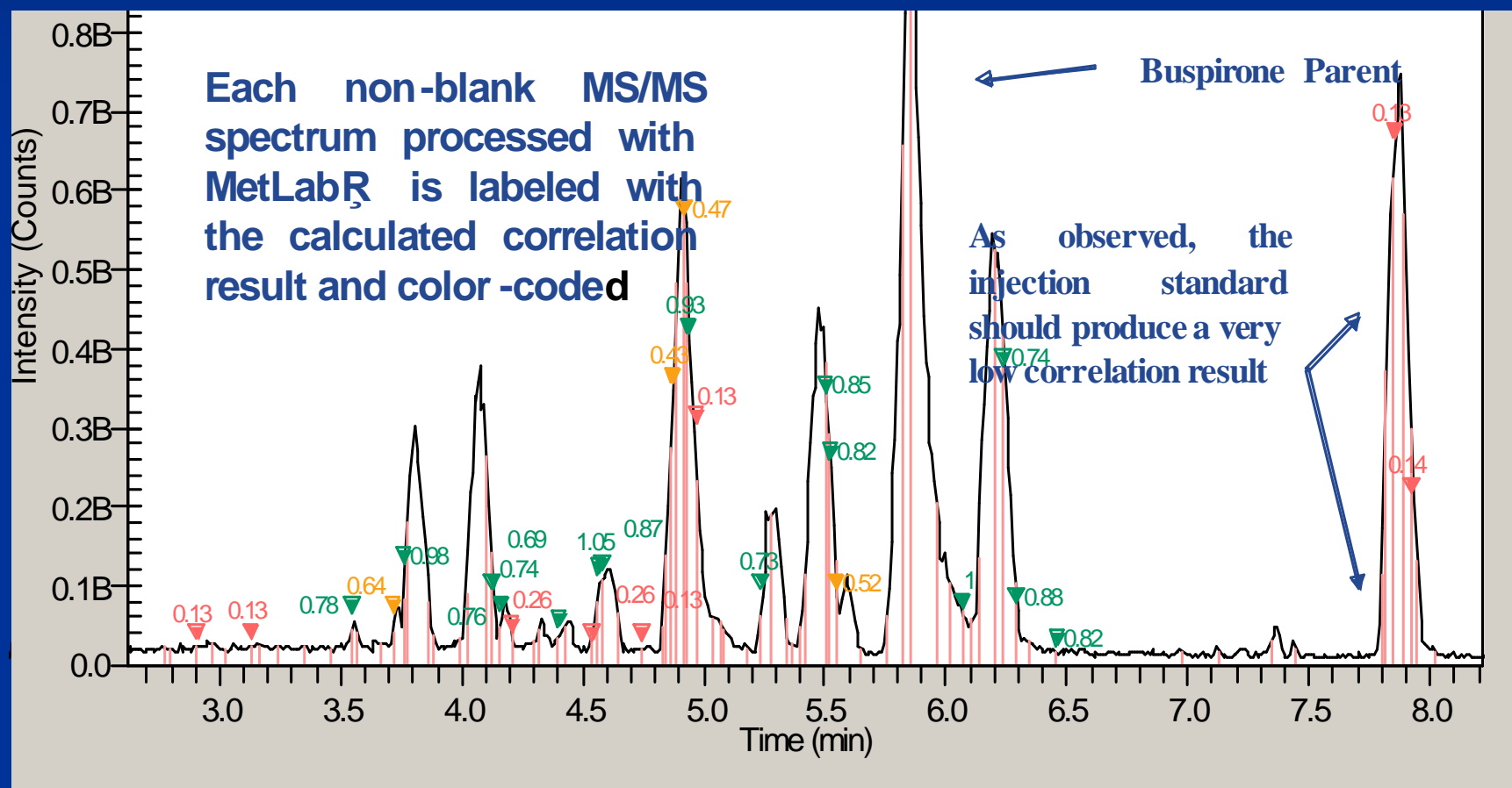
Sample Preparation:

- Incubate 700 ug with HLM (5 ml)
- Dry down to 400 ul
- Inject 80 ul on column
- Estimate metabolite @ 10 %



Buspirone Metabolite MS Profile

MetLab Identifies Related Components



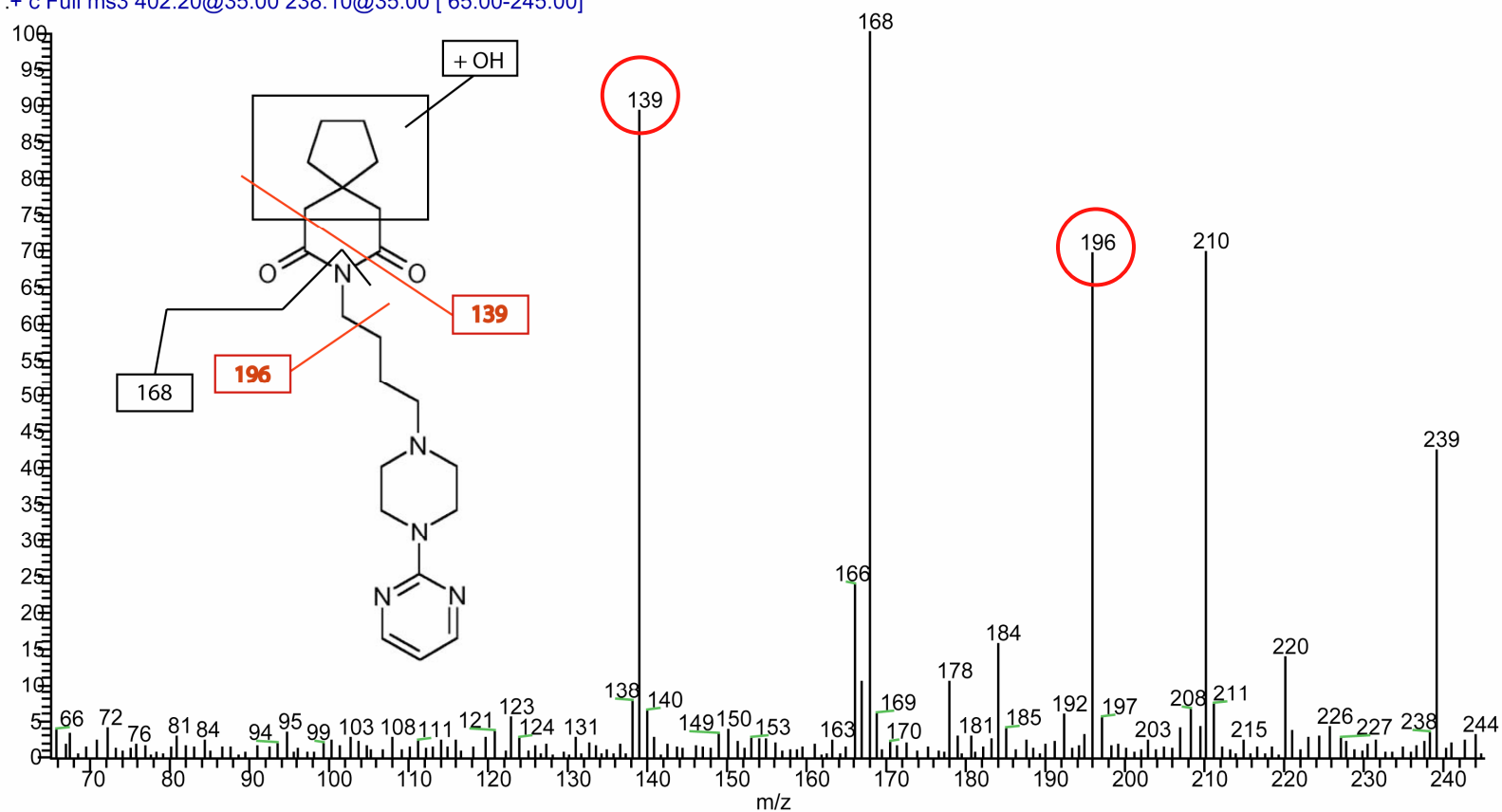
Buspirone Metabolite MS Profile

MetLab Suggests Possible Modifications

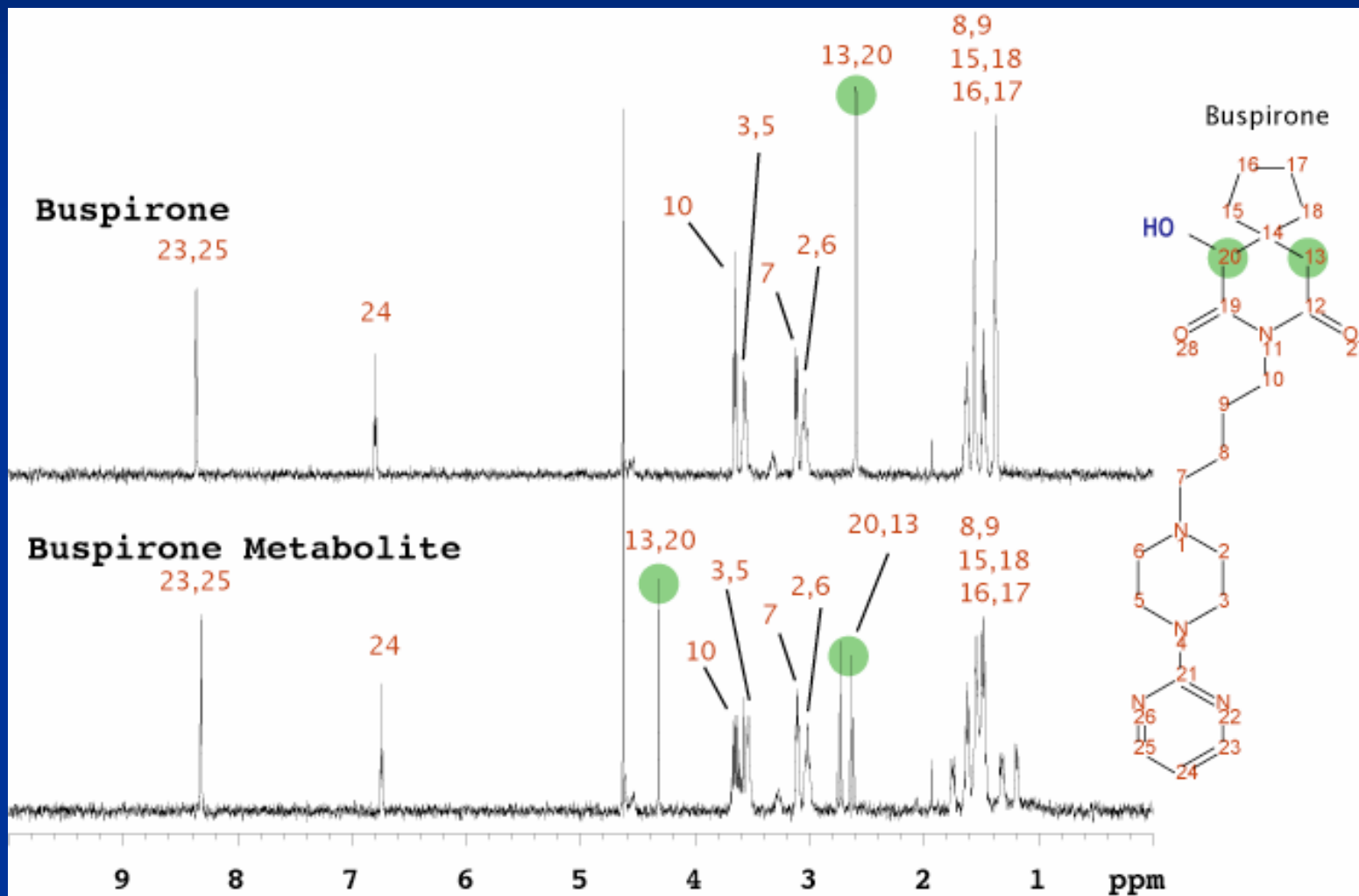
Metabolite	RT	M+H	Corr	Shifted Ions	Common Ions	Unique Ions
Dihydroxy	3.54	418	High	122(+16), 150(+16), 265(+16), 222(+16)		
Dihydroxy	3.72	418	Mid	122(+16), 150(+16), 222(+16)		235, 220
Hydroxy	3.76	402	High	265(+16), 222(+16)	122, 150	359
Hydroxy	4.12	402	High	265(+16), 222(+16), 291(+16)	122, 150	219, 220
Dihydroxy	4.16	418	High	265(+16), 222(+16)	122	267, 400, 374, 220
Dihydroxy	4.40	418	High	180(+16), 222(+16), 265(+16)		267, 164, 293, 374
Hydroxy	4.56	402	High	222(+16), 265(+16)	122, 150	359
Dihydroxy	4.58	418	High	122(+16), 150(+16), 265(+16), 222(+16)		400
Unknown	4.87	360	Mid		122, 265	343
Hydroxy	4.93	402	High	222(+16), 265(+16)	122, 150	359, 342
Dihydroxy	5.24	418	High	222(+16), 265(+16)		267, 293, 306, 374, 400
Hydroxy	5.50	402	High	122(+16), 150(+16)	222, 265, 180	
Unknown	5.52	372	High	150(-14)	122, 222, 265	251
Parent	5.86	386	High			
Hydroxy	6.24	402	High		222, 265, 122	358, 384

Buspirone Metabolite MS/MS

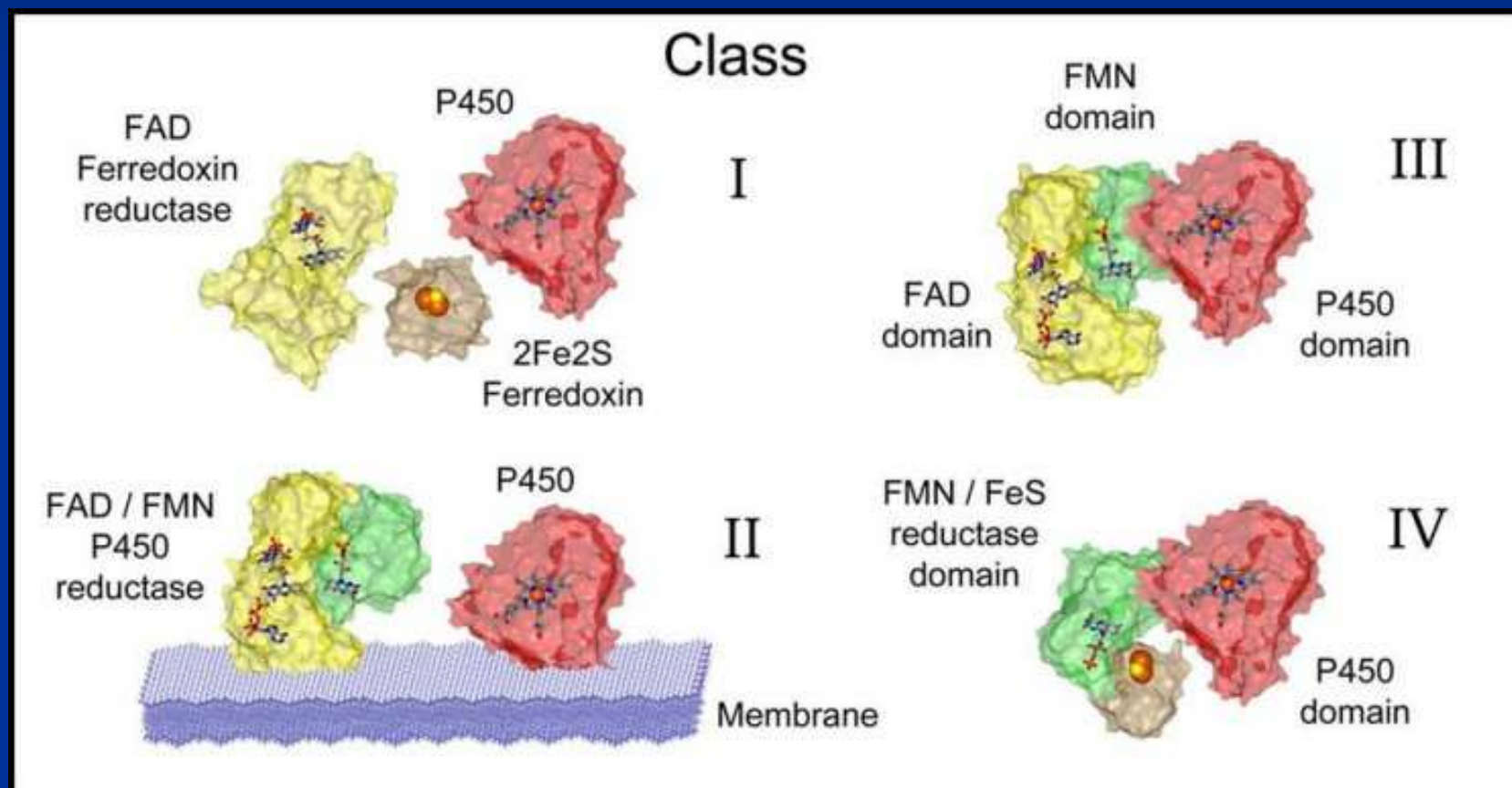
402_MS3_#213 RT:0.02-0.0AV2 NL:8.74E4
 T:+ c Full ms3 402.20@35.00 238.10@35.00 [65.00-245.00]



Buspirone Metabolite NMR



Metabolite Production (P450 Superfamily)

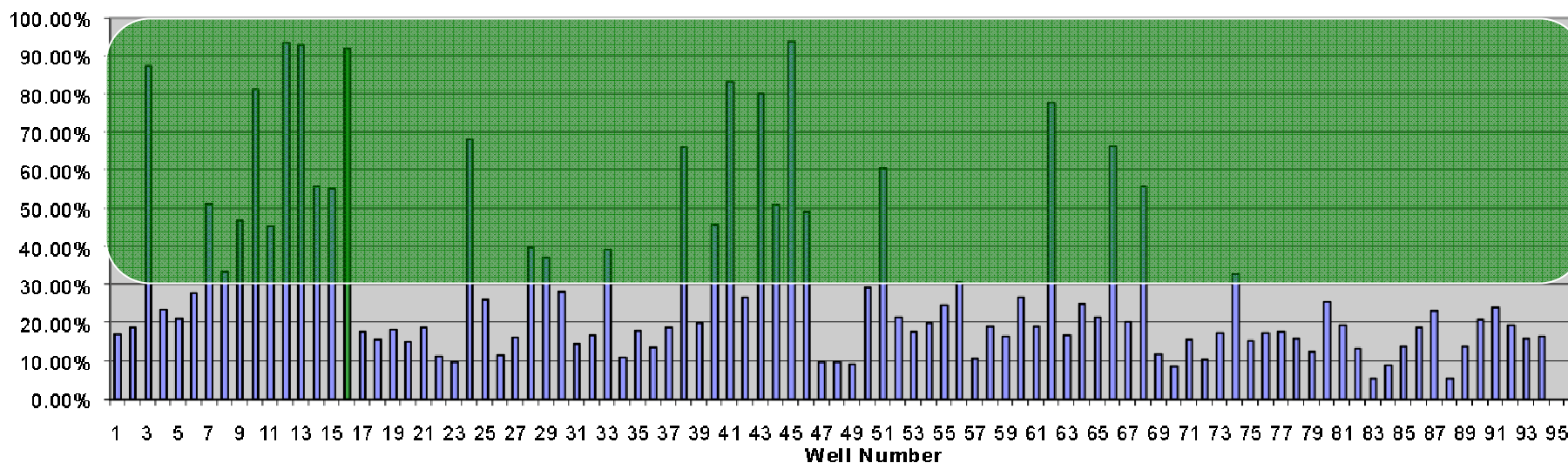


Metabolite Production

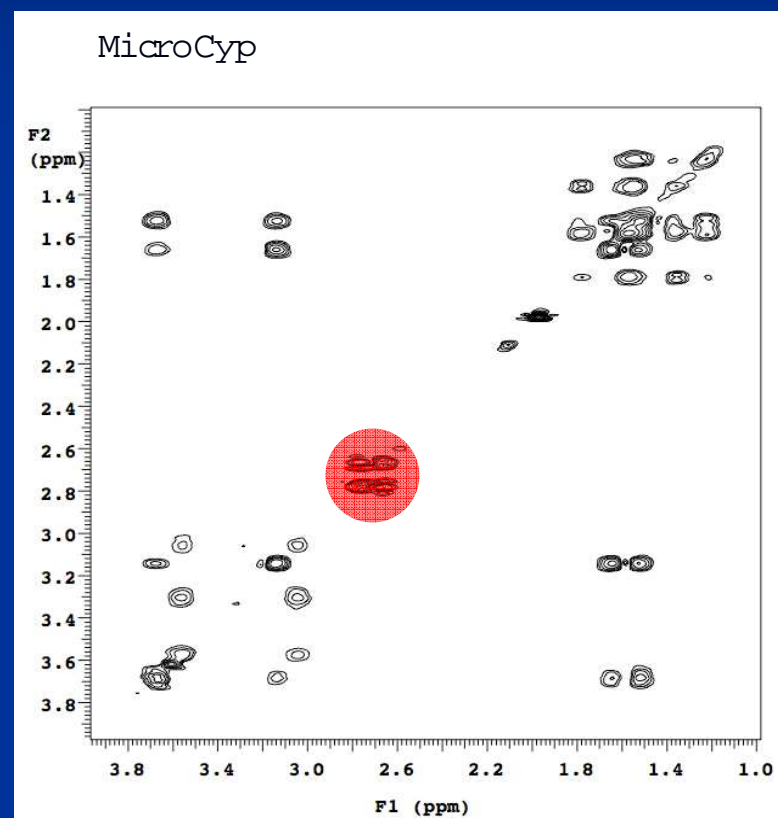
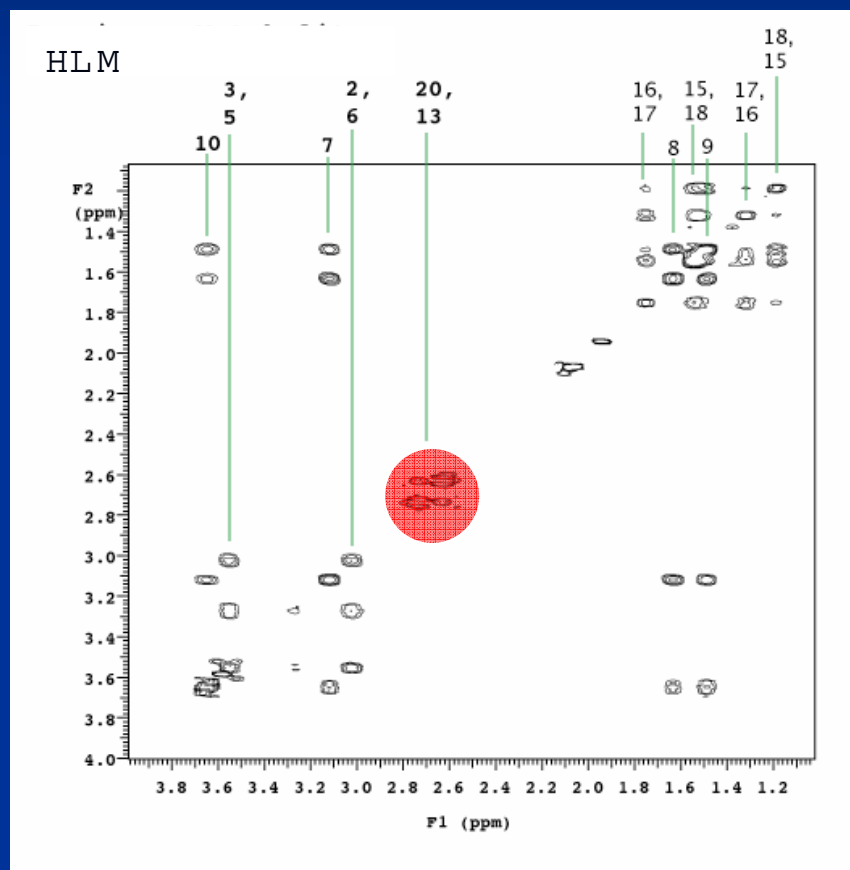
96 well plate

- Codexis MicroCyp
 - Series of GM Type III P450 Enzymes (1 per well)
 - 300 ul Incubation with 175 ug buspirone
 - 26 wells exceed 30% (50ug) of parent consumed

Percent Buspirone Metabolized



Identical Bupirone Metabolites



Conclusion & Future Developments

- Structure Elucidation Workflow
 - Full MS characterization
 - LC-NMR doesn't cut it
 - Off-line sample preparation with high sensitivity NMR is more effective

- CapNMR and SepNMR
 - CapNMR lowers sample requirements
 - SepNMR streamlines off-line sample prep
 - Currently a service
 - Working on developing into a product
 - Powerful trace analysis package

- Thanks CoSMoS 2008!