LC-NMR in Drug Discovery

Brian L Marquez, Ph.D.

Principal Scientist
Structure Elucidation Group
Pharmaceutical Sciences NMR Laboratory
Pfizer Inc.
445 Eastern Point Road, MS 8118A-2011
Groton, CT 06340

Tel: **(860) 686-1360**
Fax: **(860) 686-6227**
Email: **brian_marquez@groton.pfizer.com**

Personal Background

- Undergraduate in Biochemistry and Biophysics in 1997
- Ph.D. in Medicinal Chemistry in 2001
  - Pharmacognosy -- Marine Natural Products
- Wyeth Pharmaceuticals (PA) -- NMR Spectroscopist in Discovery
- Amgen Inc. (CA) -- NMR Spectroscopist in Discovery/PKDM/Process Chemistry
- Pfizer Inc. (CT) -- NMR Group Leader in Pharmaceutical Sciences (Development)

What we do in the Pharm. Sci. NMR group

- NMR need throughout all of drug development. This includes but is not limited to:
  - Filing characterizations
  - Lot confirmations
  - Solution confirmations
  - New technology development and integration
  - STRUCTURE ELUCIDATION
    - Impurities (e.g. process related)
    - Degradants (e.g. process related or forced degradation)

Intent of this Lecture

- What is LC-NMR – No NMR theory, just application oriented.
- Limitations
- Configurations/Options
- Practical Considerations – Overlying Theme
  - When/When not to use it

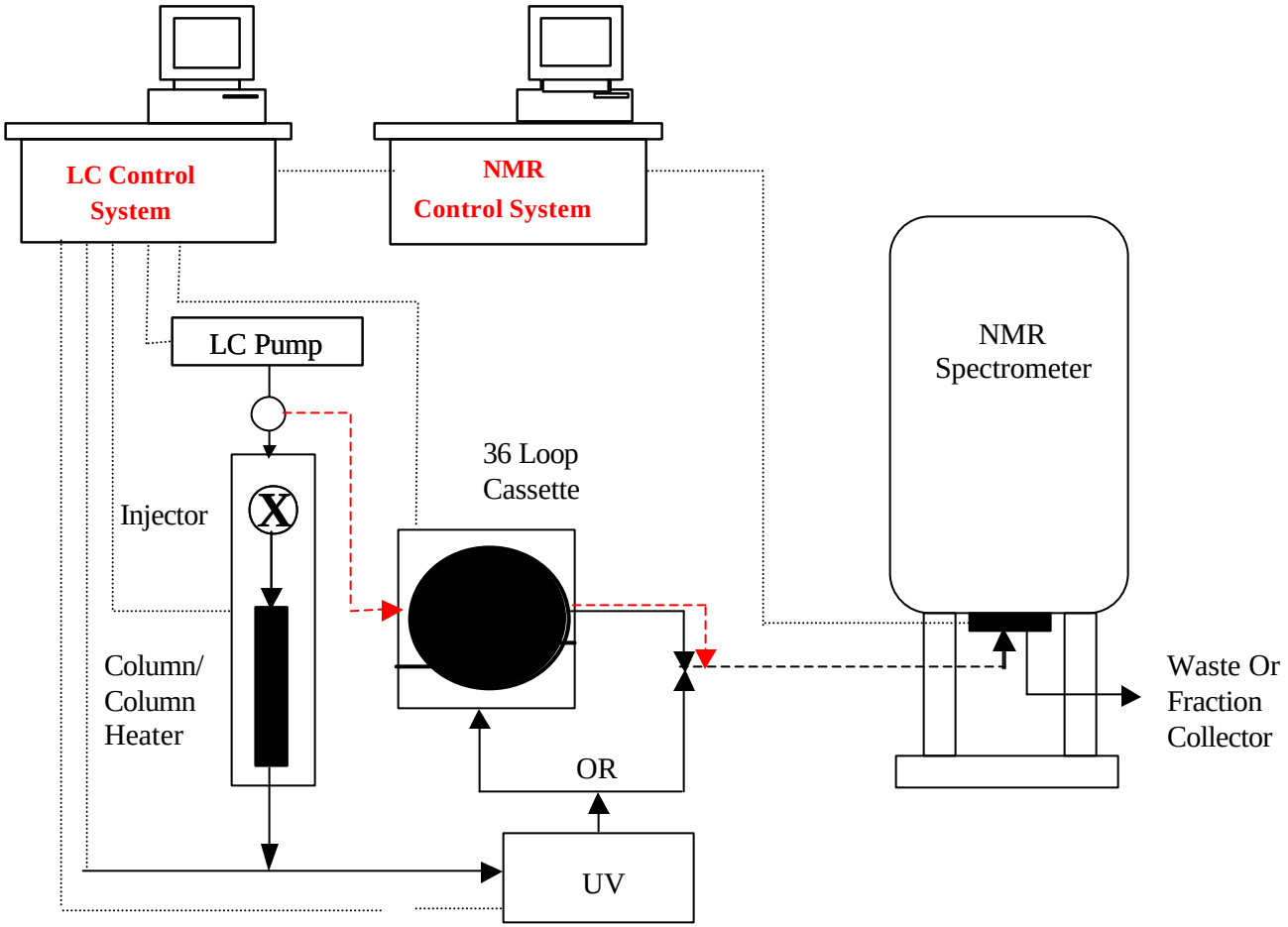
LC-NMR: Simplistic Concept

- HPLC is plumbed in line with a “flow” NMR system
- Sample components are physically separated by HPLC
- Each component flows, in turn, from the LC column and UV detector to the NMR sample cell
  - Multiple different configurations for this
- NMR is performed on each desired fraction / peak
  - Always the rate limiting step
- Continuous or stopped flow mode
  - Additional methods are also available – Peak “parking” and “trapping”.

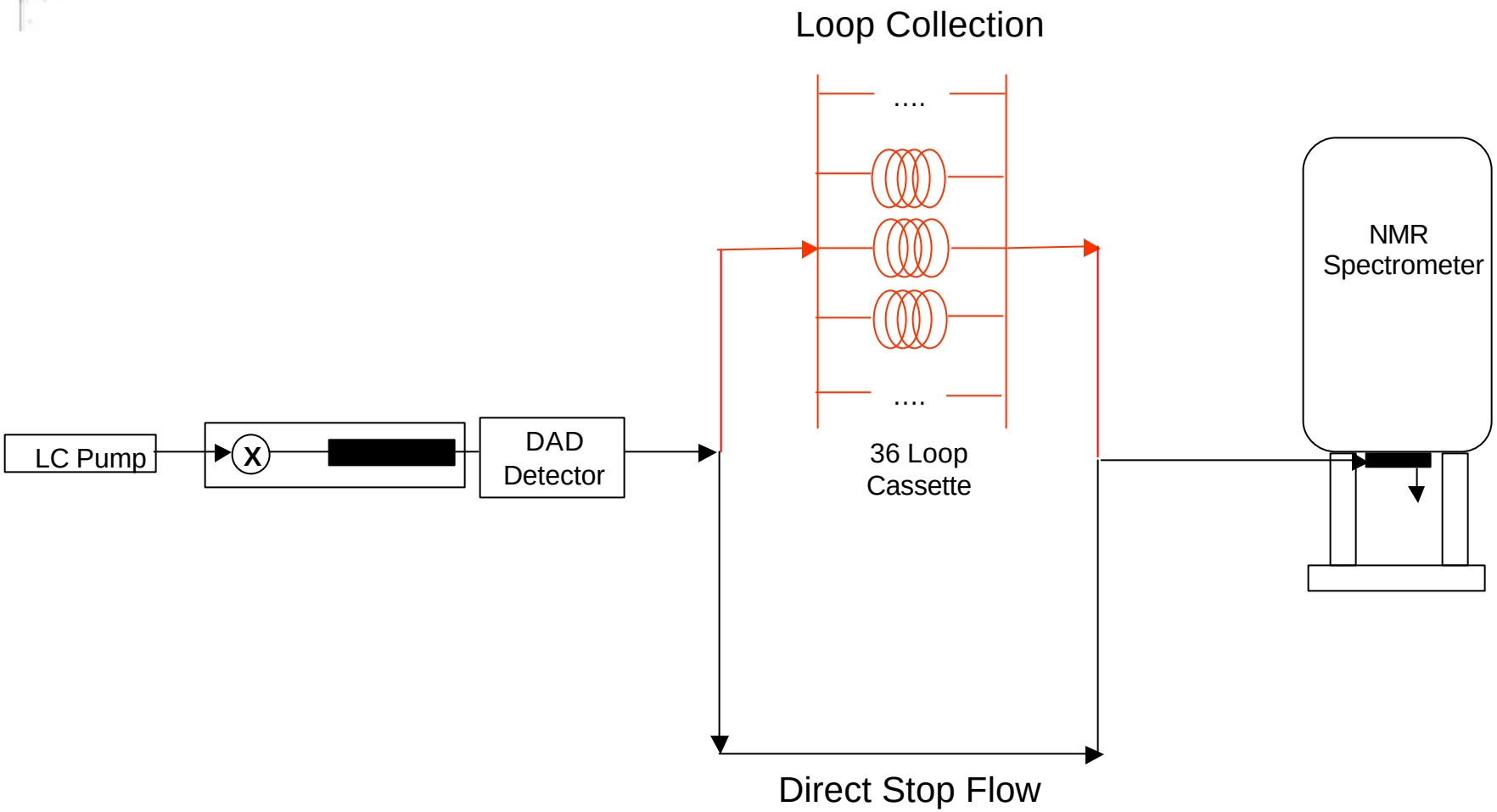
Modes of Operation

- Continuous Flow
  - Eluent sampled in “real-time” as flowing through NMR Detection Coil
- Time Slices
  - Regions, or “time-slices” of interest are analyzed
- Stopped Flow
  - Pump is stopped at desired location and data acquired
- Peak Parking
  - Peaks of interest are “parked” in off-line sample loops
- Peak Trapping
  - Solid Phase Extraction cartridges are used to “re-concentrate” samples.

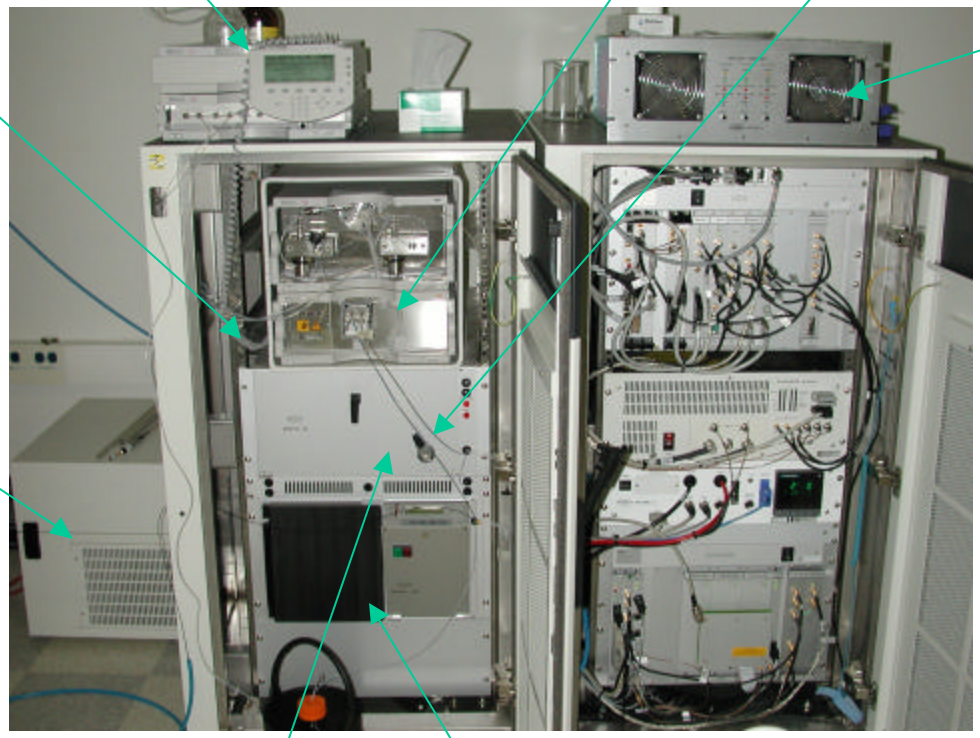
General Schematic for an LC-NMR



General Cartoon of Loop Collection

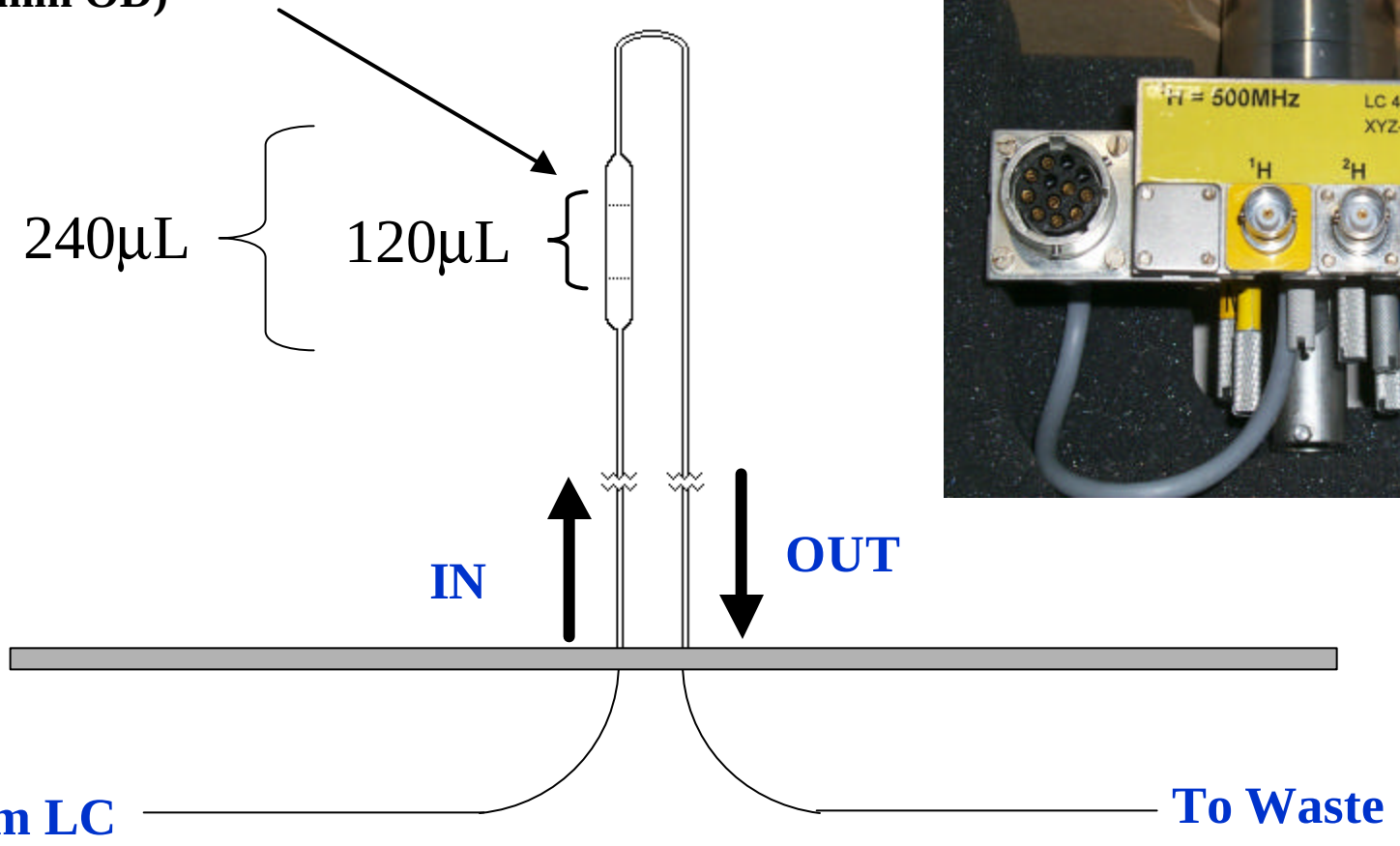


LC-NMR Hardware Configuration



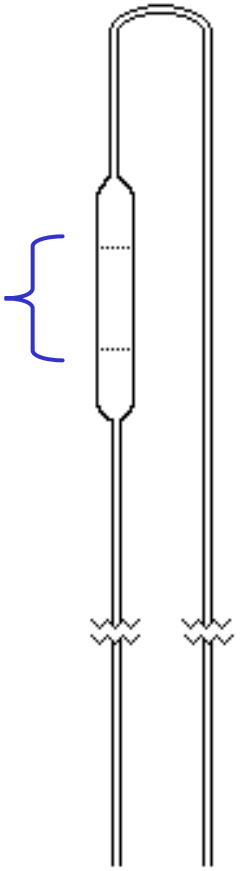
LC-NMR Probe Schematic

NMR detection coil built directly onto flow cell (4mm OD)



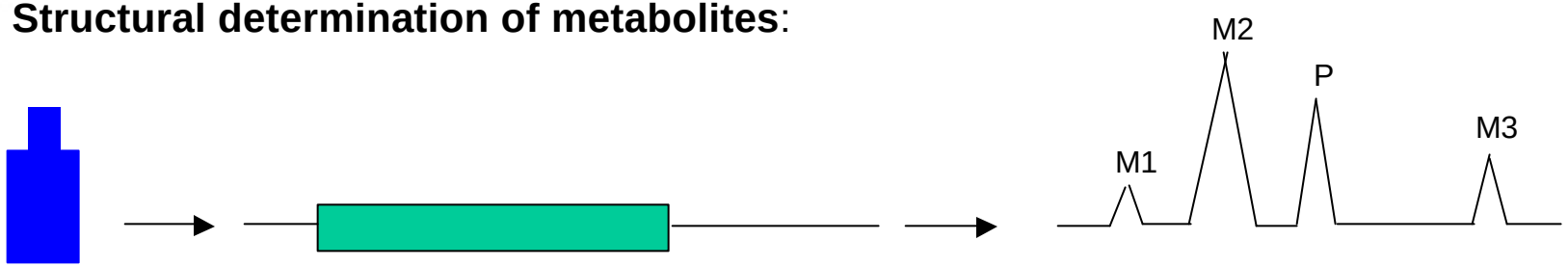
Traditional Probe Configurations

- Most common configuration – inverse probes – best proton sensitivity
- Flow Cells – Active Volumes
  - 3mm – 60µL
  - 4mm – 120µL
  - 5mm – 240 µL
  - Others “non-traditional” will be covered later
- Typically probes are outfitted with z-gradients
  - For gradient experiments and shimming



General Application Strategy

1. Structural determination of metabolites:

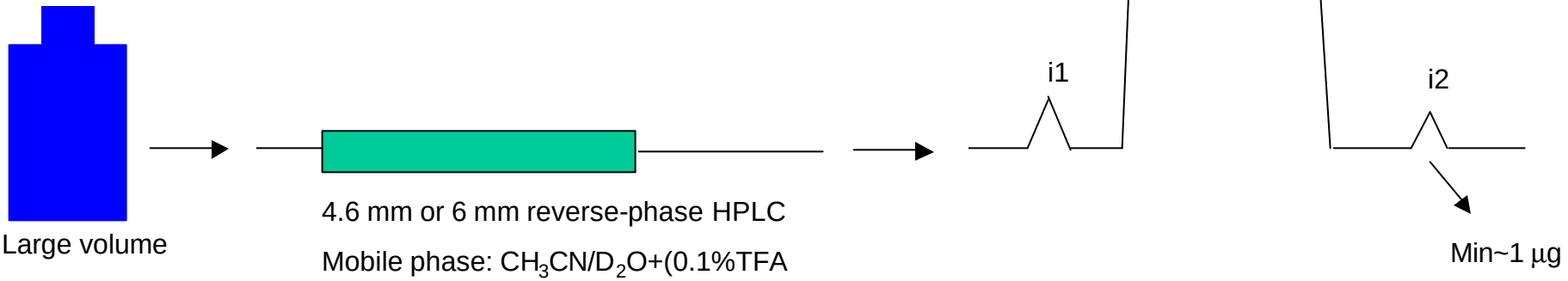


~50 µg of metabolites
From microsomal
incubations

4.6 mm or 6mm Reverse-phase HPLC

Mobile phase: CH_3CN/D_2O+(0.1%TFA or DCOOD), solvent gradient < 2%/min

2. Structural determination of impurities:



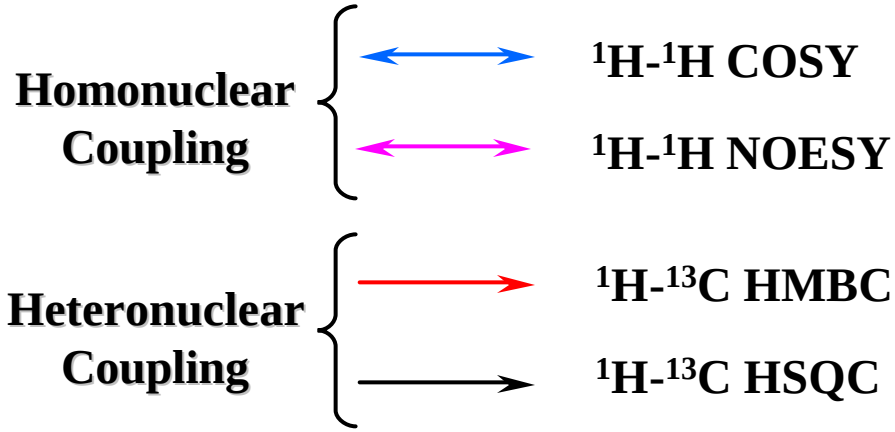
Large volume
injections in high % of
aqueous phase
More then 1 mg

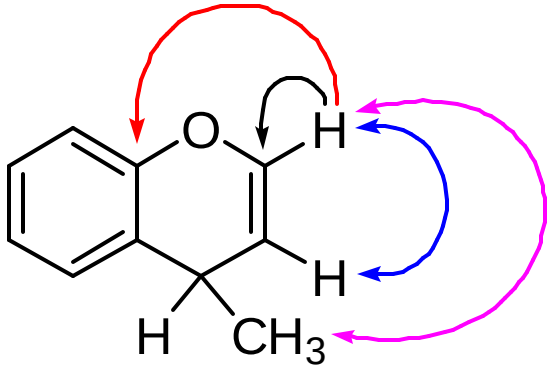
4.6 mm or 6 mm reverse-phase HPLC

Mobile phase: CH_3CN/D_2O+(0.1%TFA or DCOOD), solvent gradient < 2%/min

Min~1 µg

Structure Elucidation Using NMR





- 1D ^{1}H and homonuclear NMR experiments are the most sensitive and accessible experiments for LC-NMR
- 1D ^{13}C and heteronuclear NMR experiments are very insensitive and are typically inaccessible to LC-NMR applications (in most cases).

When to use LC-NMR

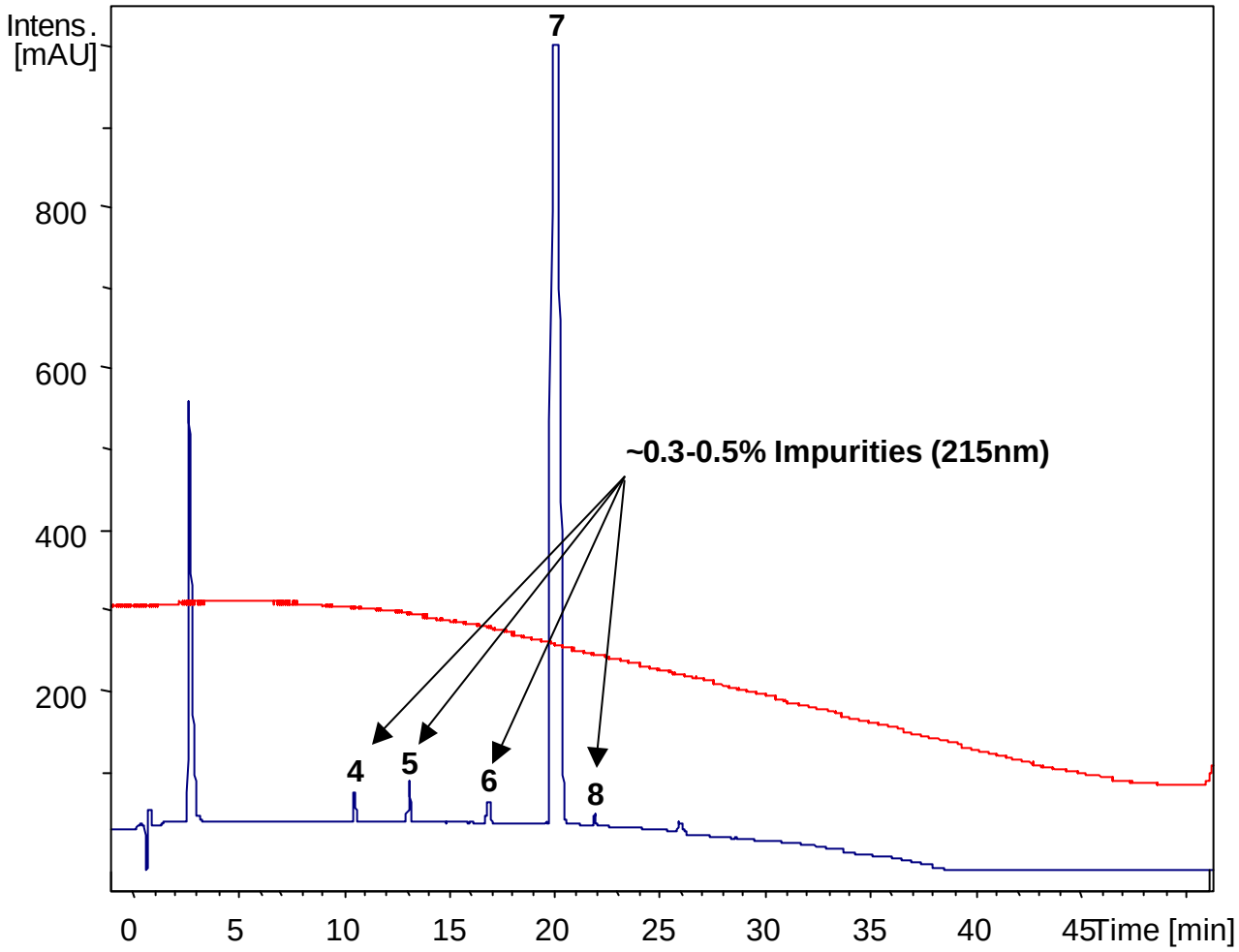
- Fairly resolved peaks.
- Relative abundance of entities similar.
- Known stability issues.
- A significant amount of information is known about the compound

Impurity Analysis: Low Volume (20µL) Injection of Sample

20µL injected

t	%B
0	10
48	100
50	100
50.5	10
51	10

1.5mL/min
4.6x150mm Luna 5u C8(2)
A: 0.1% TFA/$D_2$0
B: 0.1% TFA/MeCN



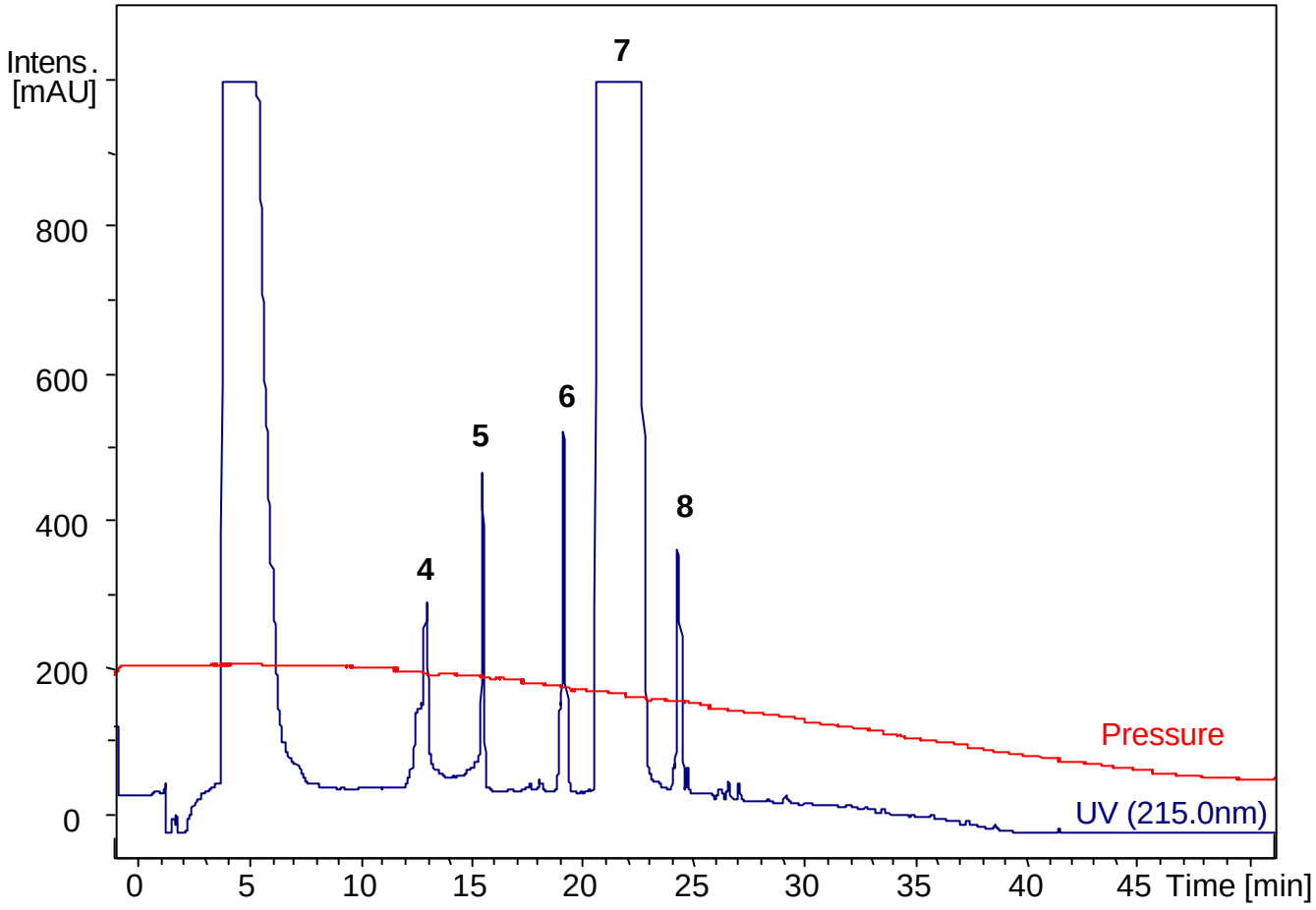
In order to achieve sufficient NMR sensitivity it was necessary to overload the HPLC column without sacrificing peak resolution. This goal was achieved by maximizing sample concentration in the highest content of aqueous phase followed by large volume column injections.

Impurity Analysis: Large Volume (500µL) Injection of Sample

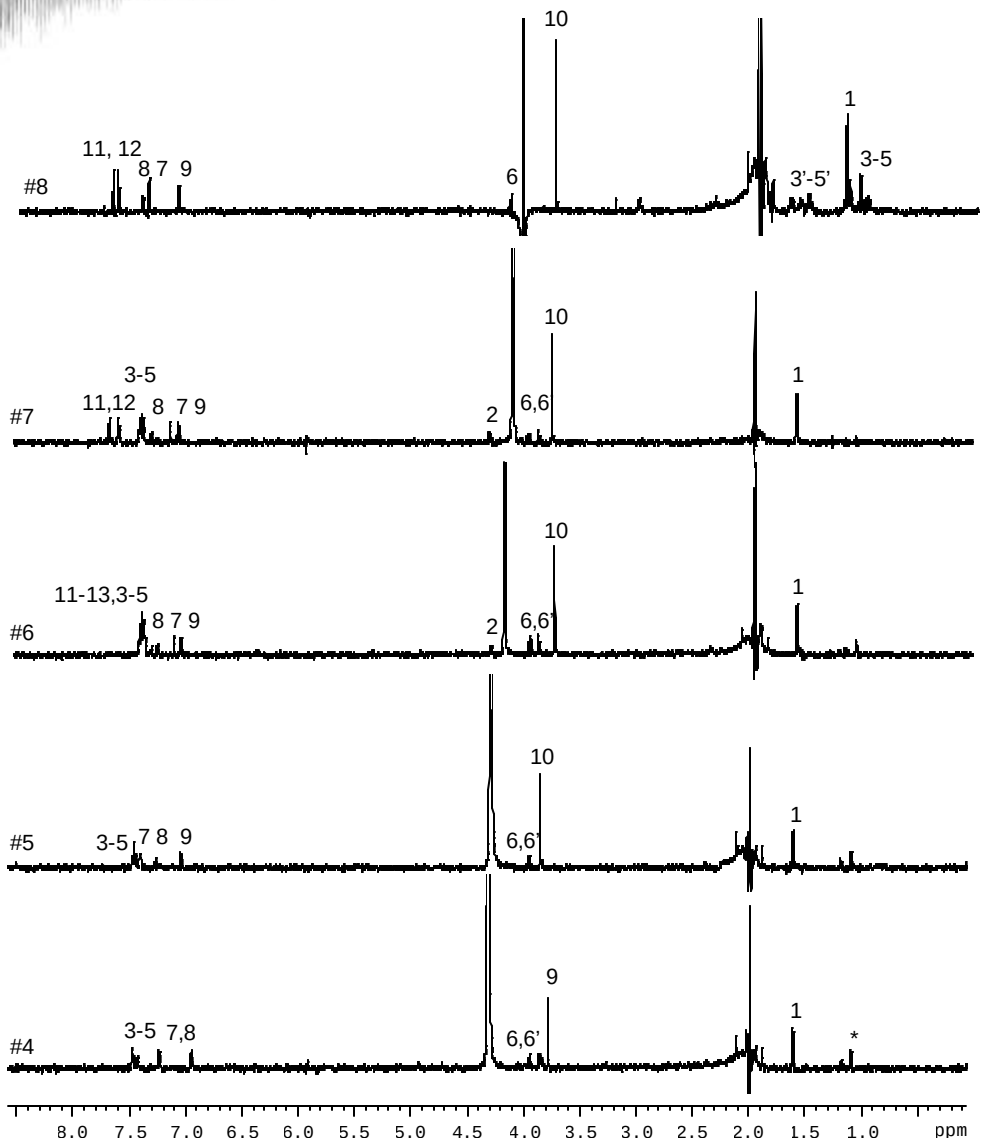
500µL injected

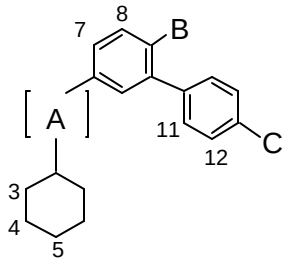
t	%B
0	10
48	100
50	100
50.5	10
51	10

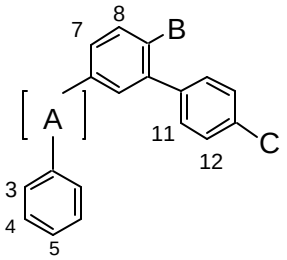
1mL/min
4.6x150mm Luna 5u C8(2)
A: 0.1% TFA/D_2O
B: 0.1% TFA/MeCN

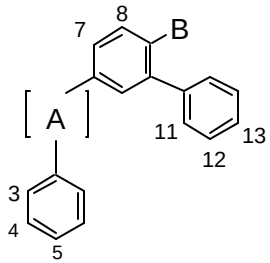


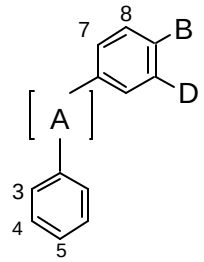
Impurity Analysis: Solved Structures

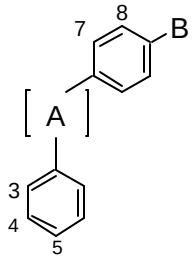










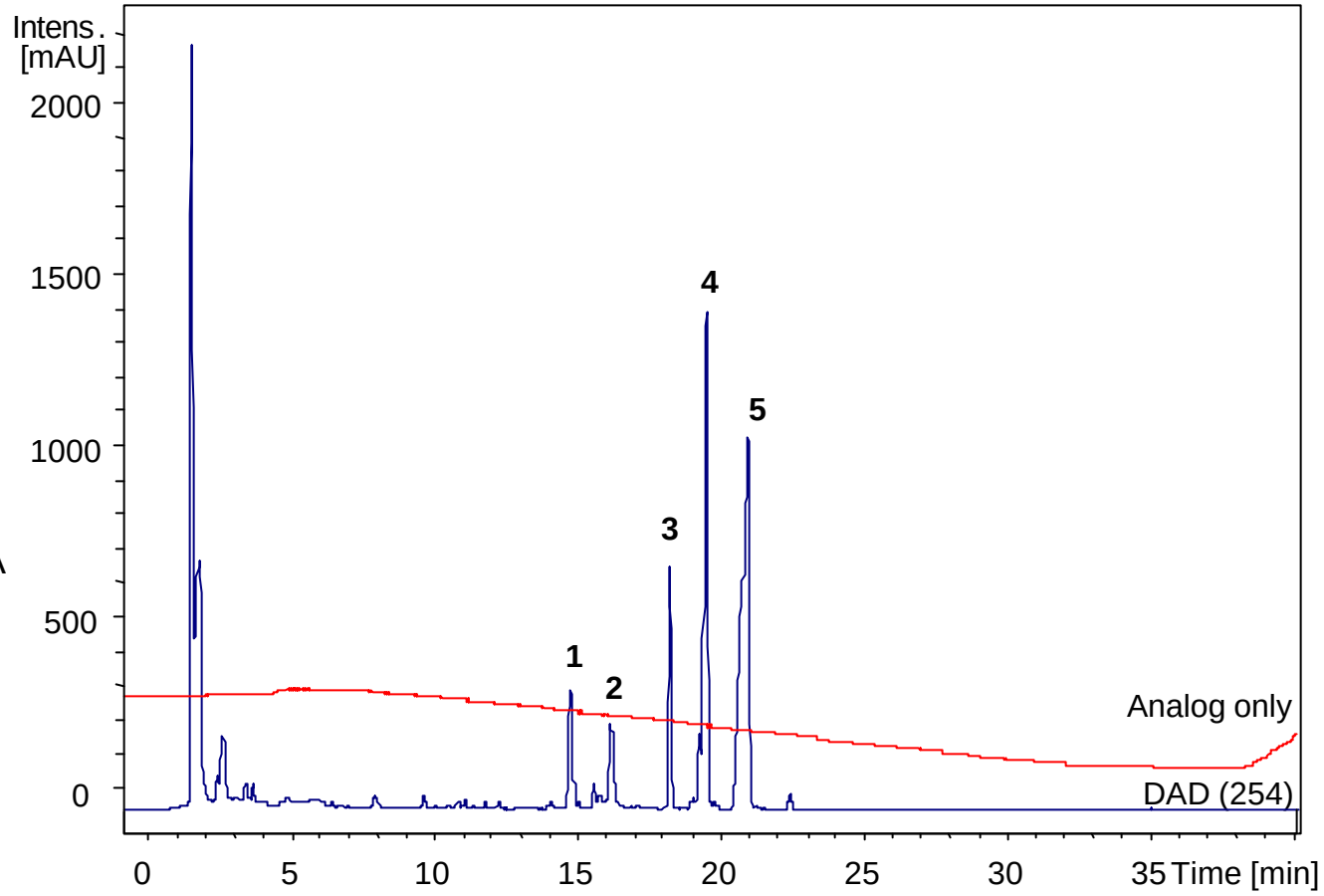


Metabolite Analysis:

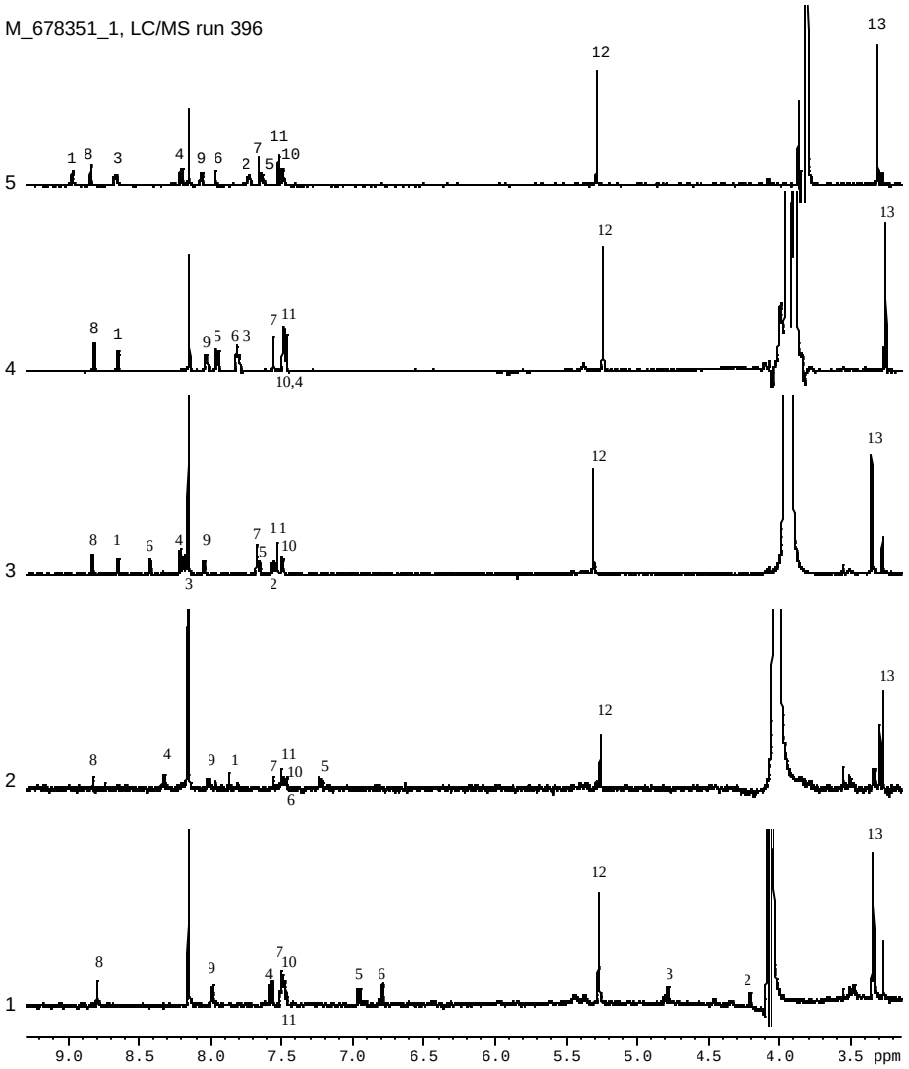
100µL of ~4mg/mL injected

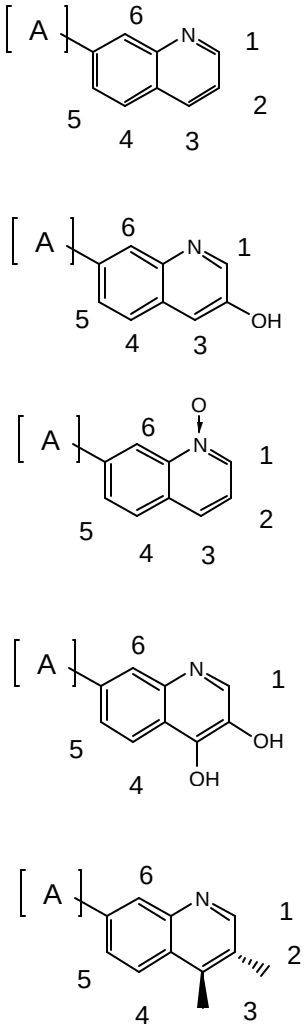
t	%B
0	35
5	35
30	95
37	95
38	35
40	35

1.0mL/min
4.6x250mm YMC-AQ 5u 120A
A: 0.1% d-FA/$D_2$0
B: 0.1% d-FA/MeCN



Metabolite Analysis: Solved Structures



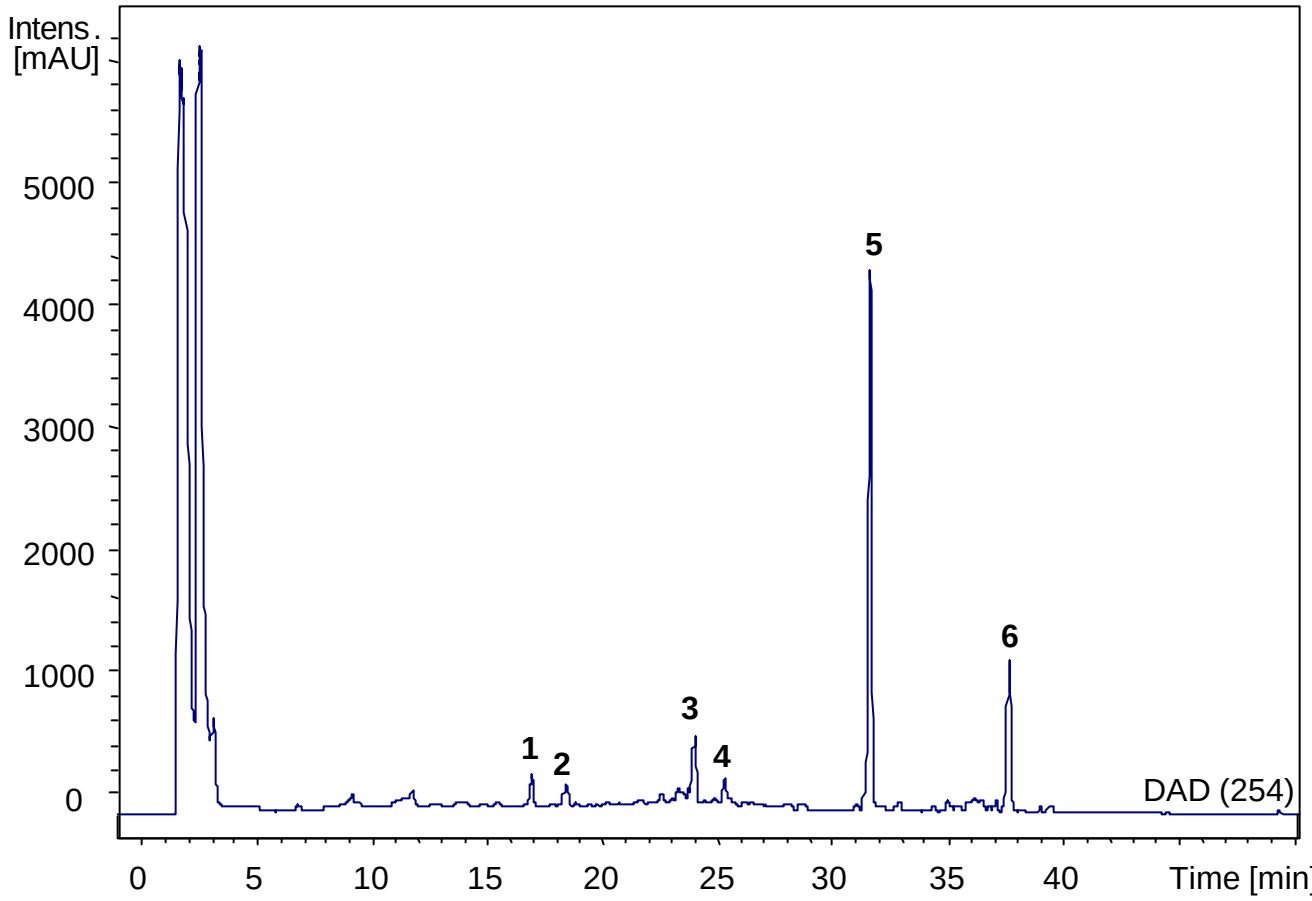


Metabolite Analysis:

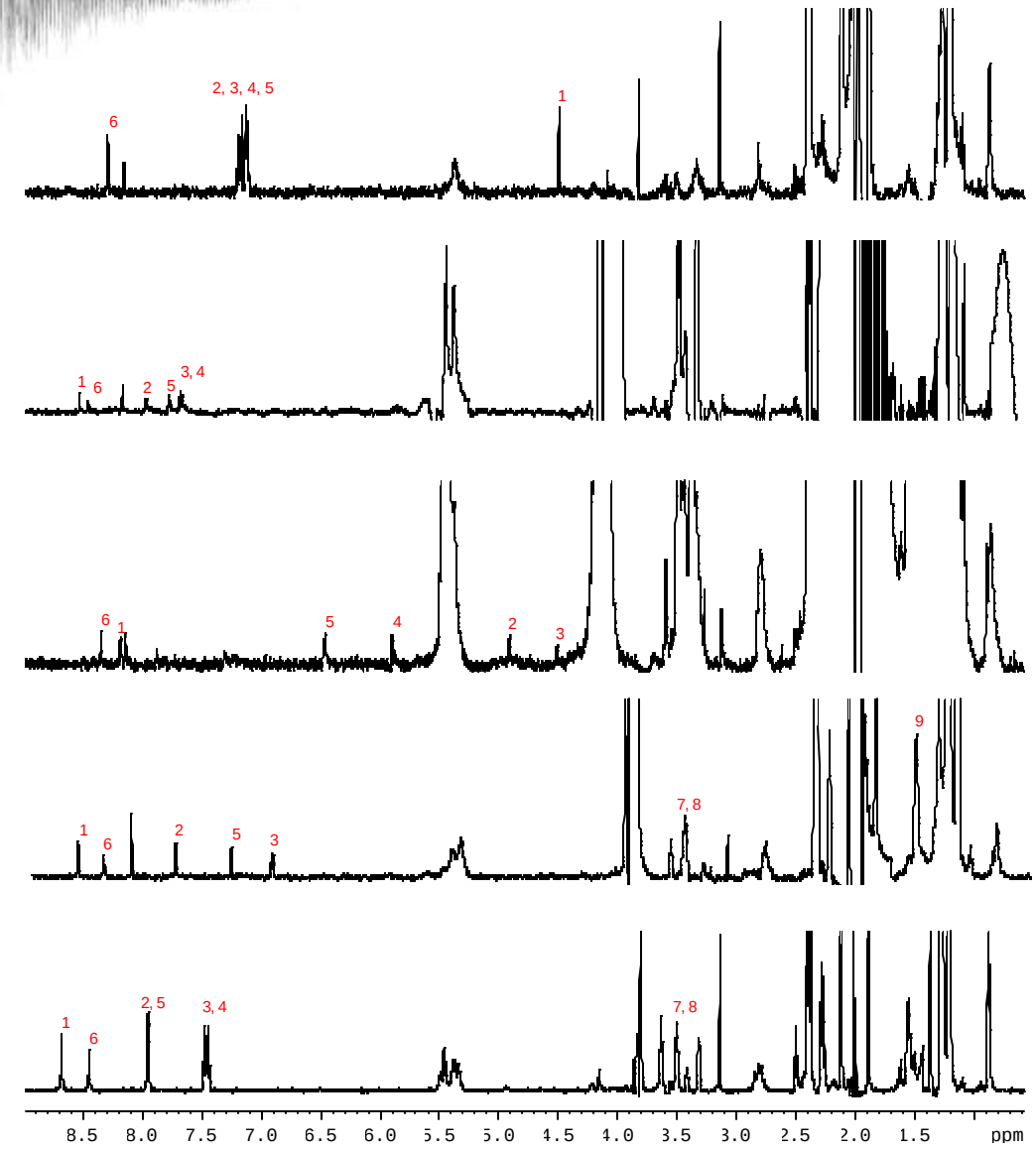
500µL injected

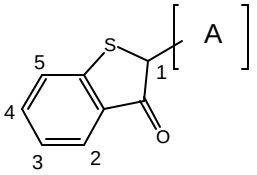
t	%B
0	20
2	20
40	90
45	90
46	20
50	20

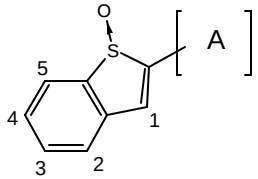
1.0mL/min
6x150mm YMC-AQ 3u 12n
A: 0.1% d-AcOH/$D_2$0
B: 0.1% d-AcOH/MeCN

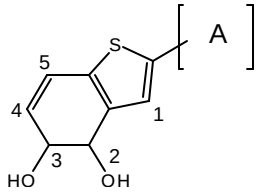


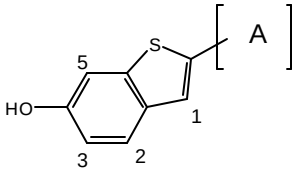
Metabolite Analysis: Solved Structures

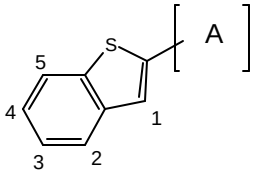




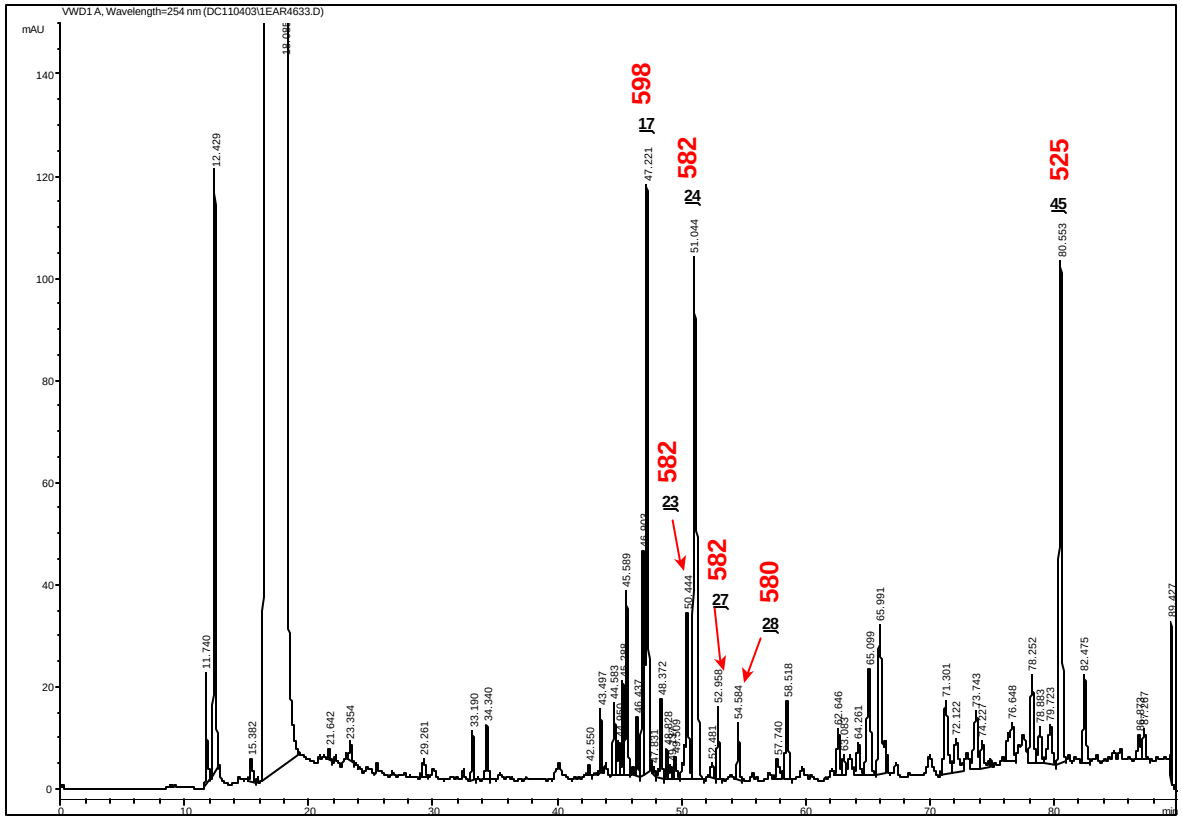






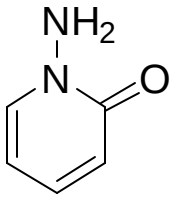


When not to use LC-NMR

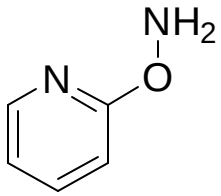


Regiochemistry

- Sample submitted for determination of the regiochemistry of the primary amine moiety



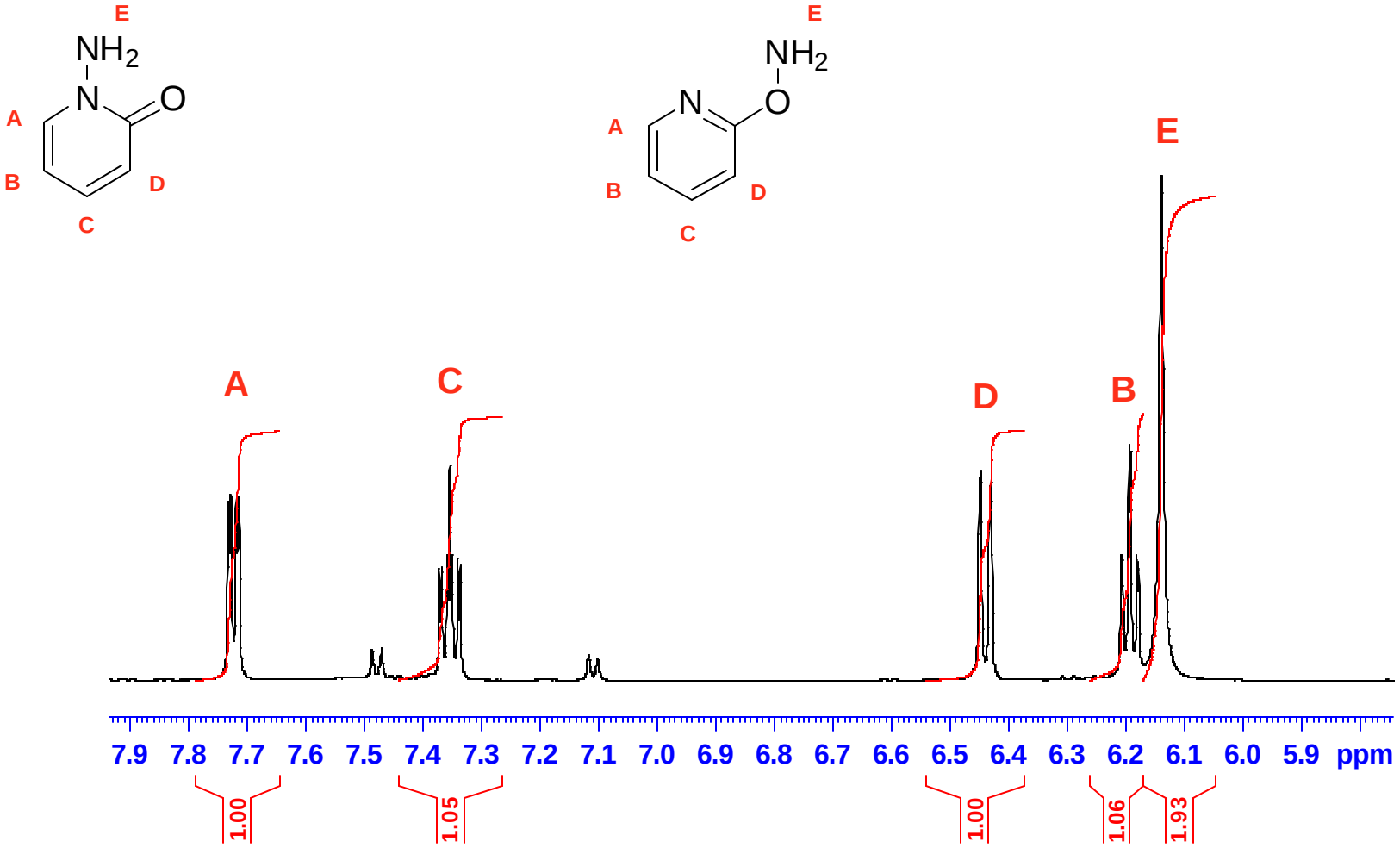
Structure A



Structure B

^{1}H Assignment

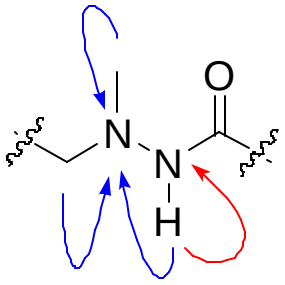
Very little help using Chemical Shift Arguments



^{1}H-^{15}N HMBC Data

Background -- Simplified

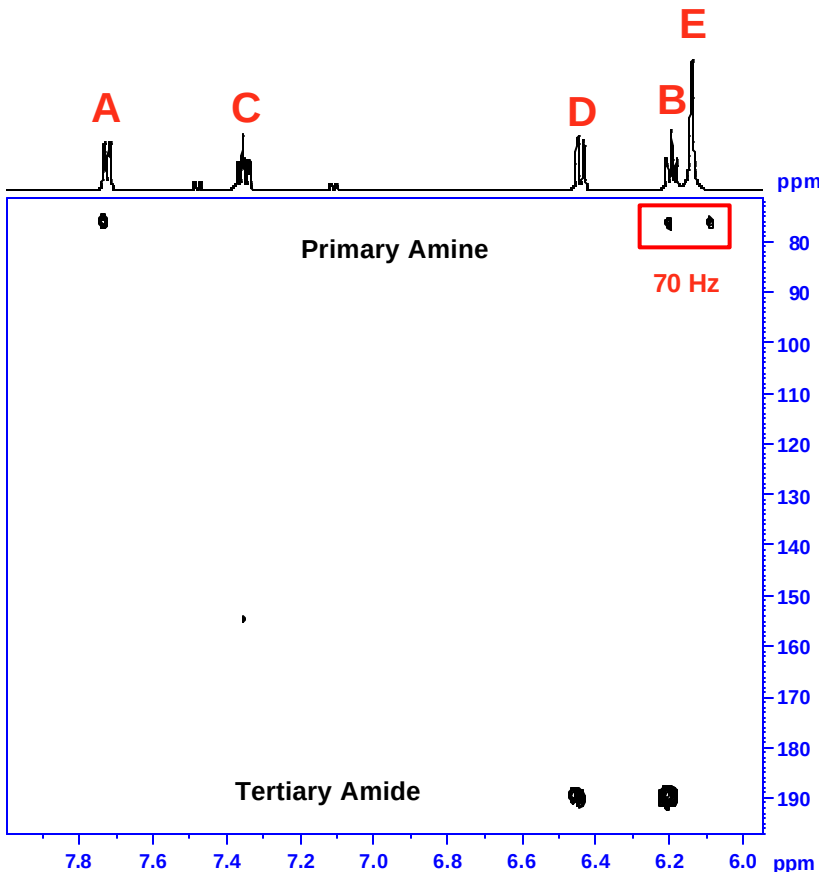
- Pulse sequence allows one to detect ^{1}H's long-range coupled (~8 Hz) to ^{15}N
  - Depending on the experiment used one can choose to omit or retain the $^{1}J_{NH}$

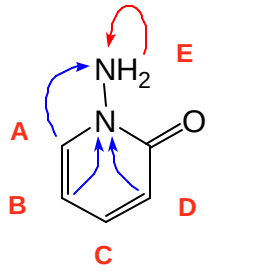


→ $^{n}J_{NH}$ (n = 2,3) ? Will result in a correlation centered at the ^{1}H and ^{15}N chemical shift

→ $^{1}J_{NH}$? Will result in a correlation centered at the ^{15}N chemical shift and a split signal centered on ^{1}H

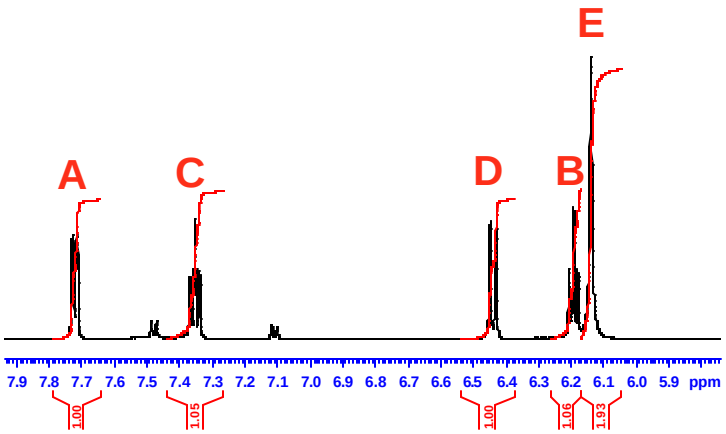
^{1}H-^{15}N HMBC Data Allows Assignment





$^{n}J_{NH}$ (n = 2,3)

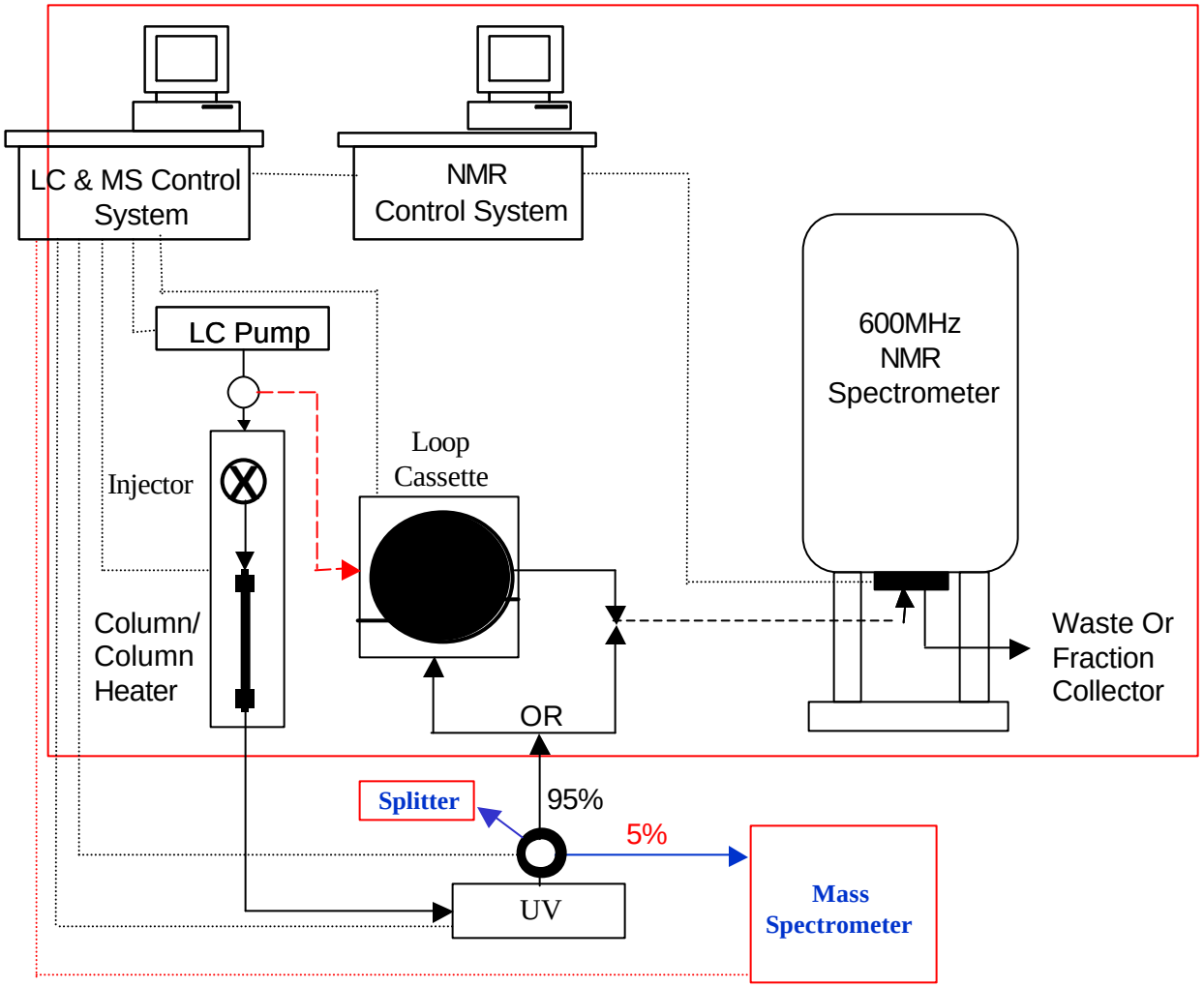
$^{1}J_{NH}$



Other Options Available

- LC-NMR/MS
  - Allows on-line MS and NMR evaluation of samples
- Peak Trapping (Column Trapping)
  - Potentially allows multiple LC peaks to be “trapped” and concentrated prior to NMR data acquisition.
- Microcoil Probes
  - Has potential to allow microscale separation mechanisms (e.g. CapLC).
- CryoProbe Technology
  - Significantly lowers “noise floor” through cryo-cooling RF electronics in the probe.

LC-NMR/MS



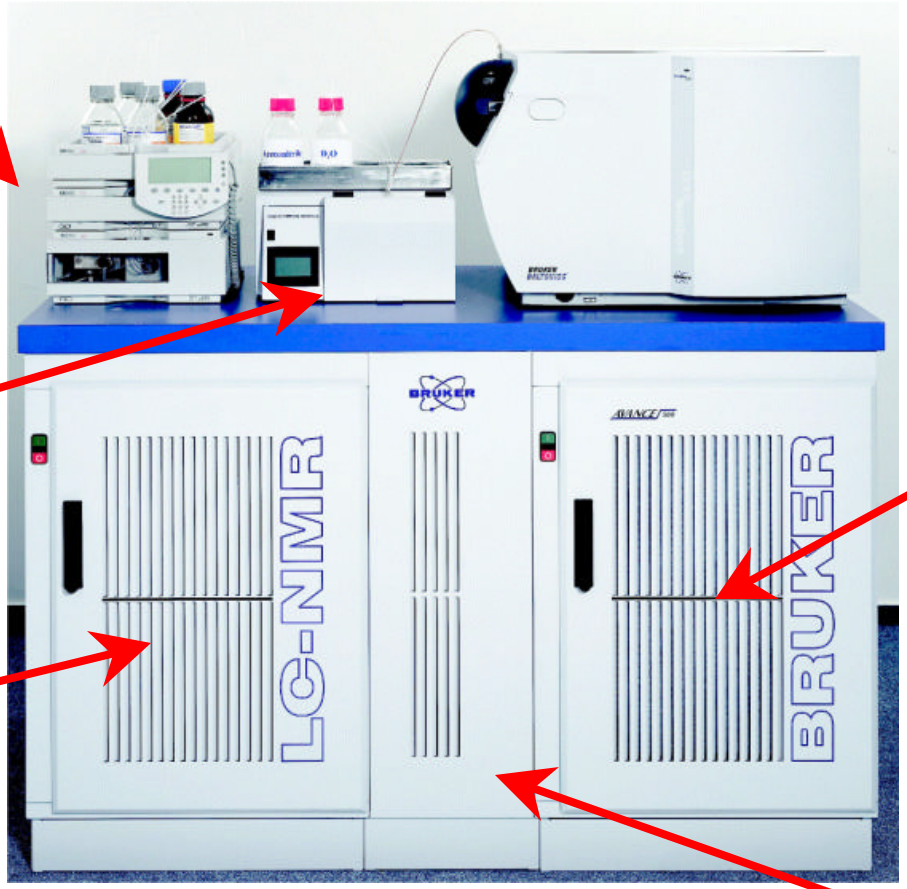
MS Component

Actual System

Hardware Setup for LC-NMR-MS (without magnet)

HPLC

Esquire Ion Trap
Mass-Spectrometer



LC-NMR-MS Interface
BNMI

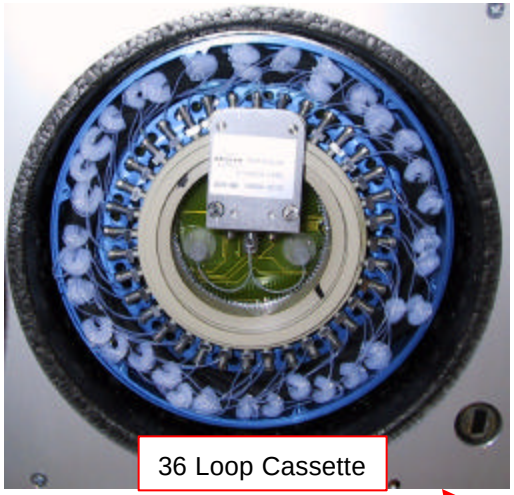
NMR Spectrometer
electronics

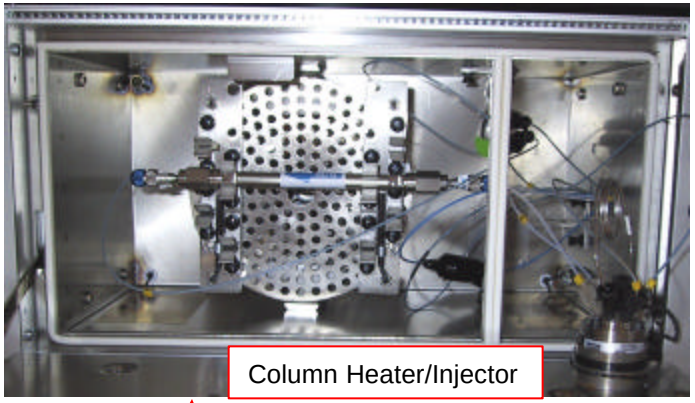
UV detector
BPSU-36/
BSFU-O

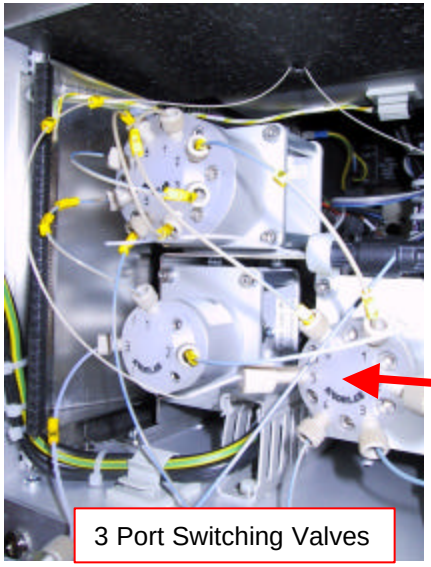
MS-Rough pump

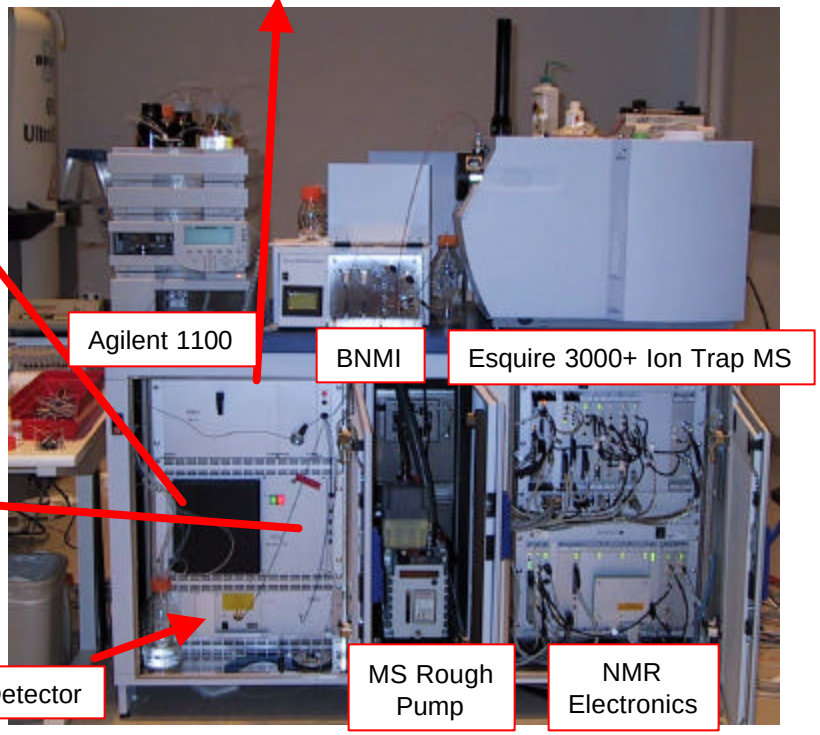
Graphic Borrowed from Bruker-Biospin

Actual System



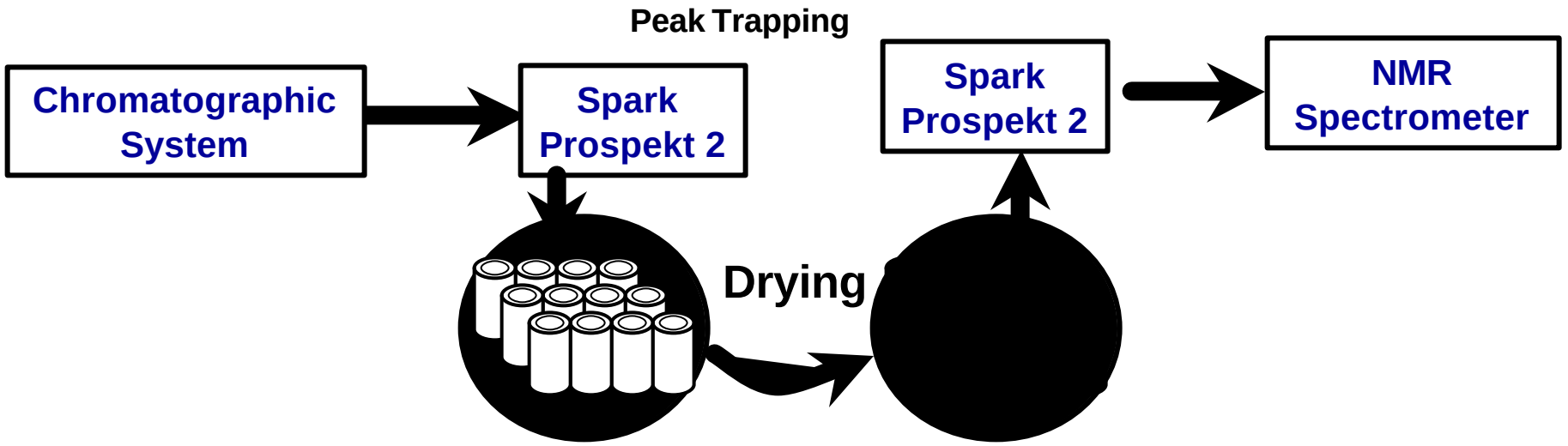




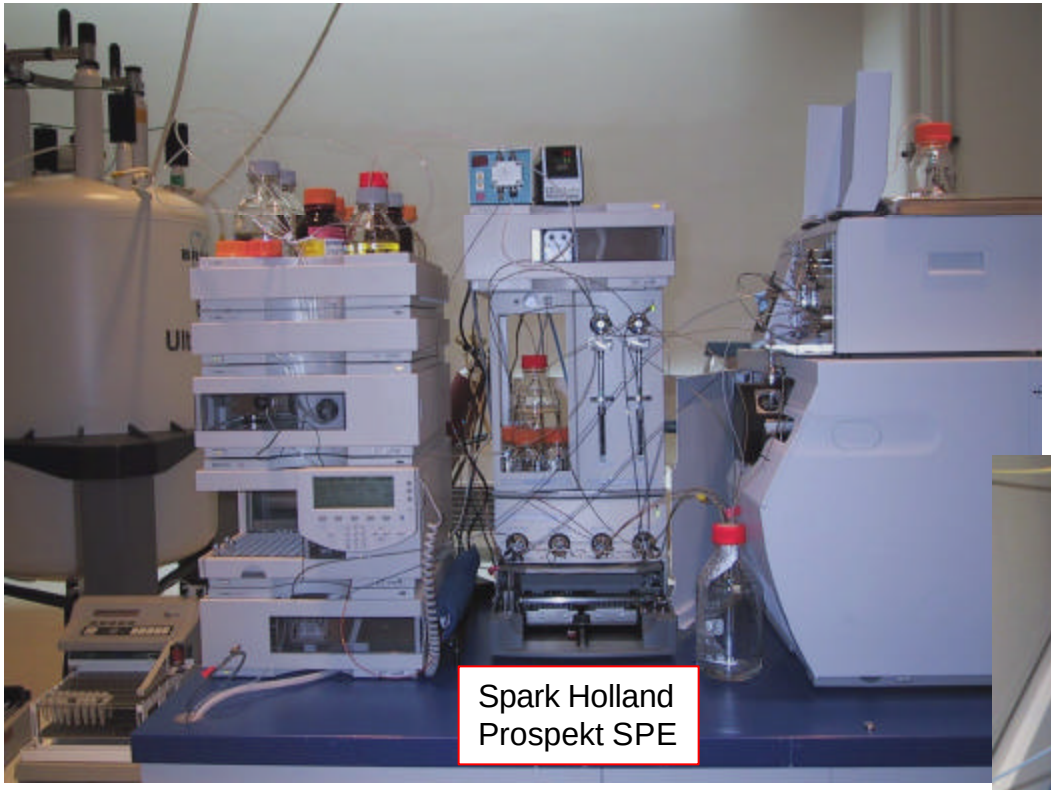


Other Options

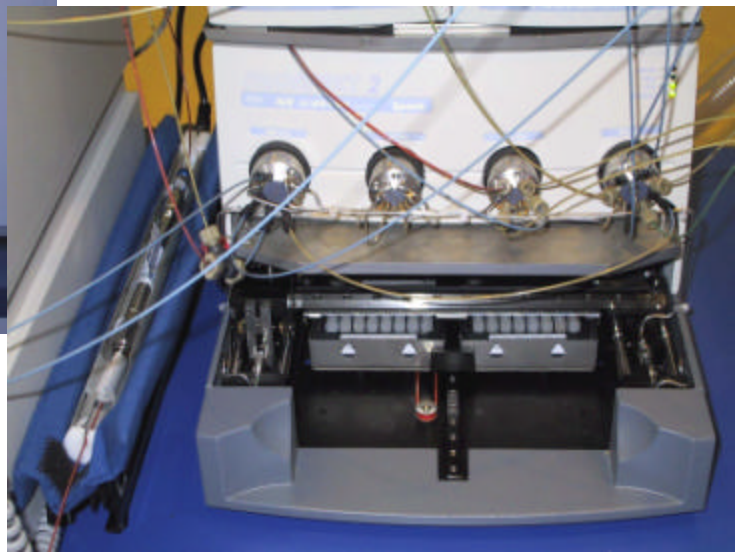
LC-SPE-MS-NMR



Closer Look at the SPE



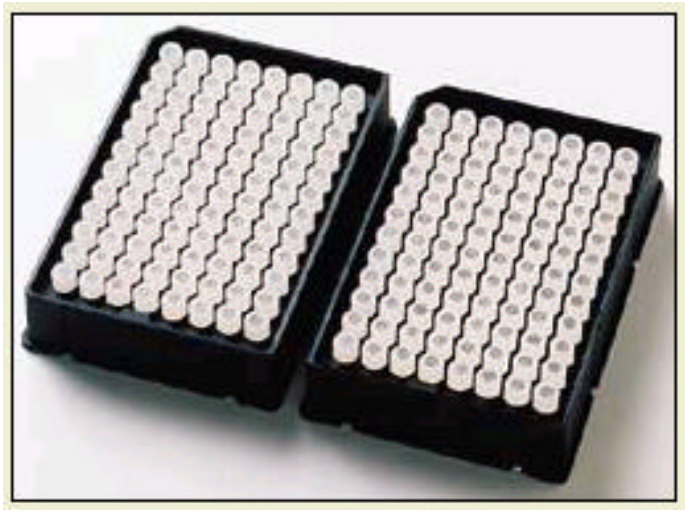
Spark Holland
Prospekt SPE



Robot gripper for SPE cartridges

2 flow lines where trap cartridges are inserted

SPARK HOLLAND SPE-UNIT



Trap cartridge size
10mm * 2mm (ID)
10mm * 1mm (ID)
10mm * 3mm (ID)

~ 2 $ per cartridge

Various commercial packings available

Bruker provides a set of 4 different solid phase types to start

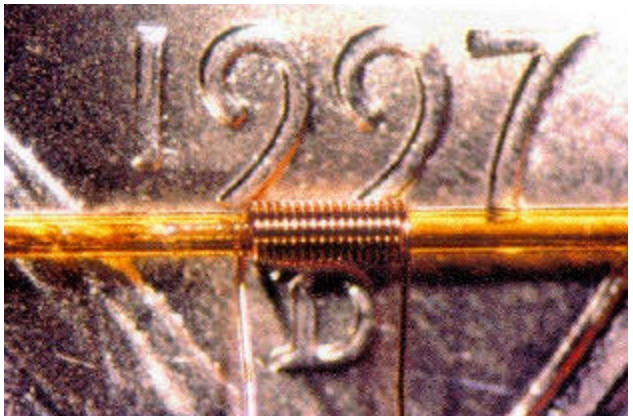
Graphic Borrowed from Bruker-Biospin

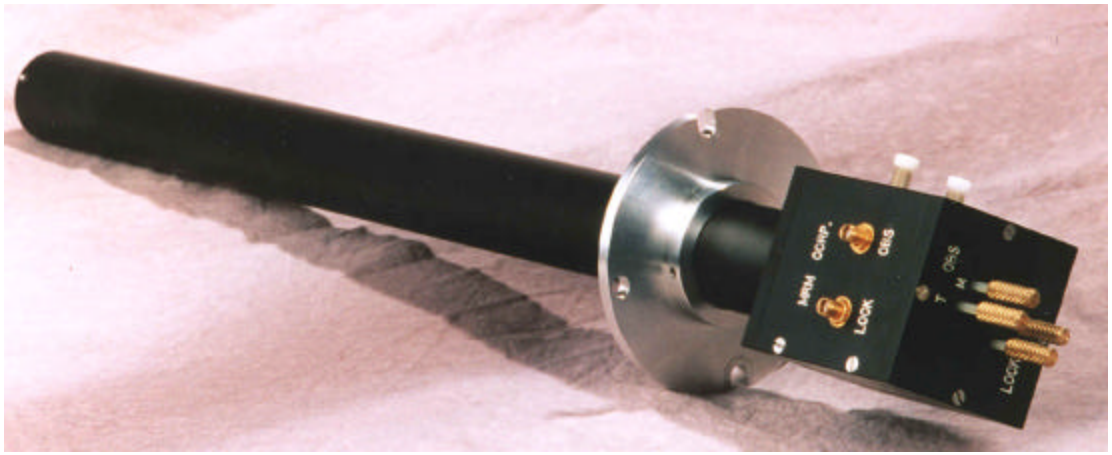
Major New Developments

- Miniaturization – Microcoil Probes
- Integration of new (to commercial NMR) MS
- Cryogenic NMR Flow Probes

MicroCoil Probe

- Horizontal copper RF solenoid Coil
- Vertical (Z) pulse field gradient (PFG) coil
- Flow cell is surrounded by CF-43 fluorocarbon for susceptibility matched to copper coil
- 1.5 μL active volume with a 5 μL total volume
- 7 μL total volume from inlet to outlet (3 μL transfer from injection assembly)
- Lock power > 45 db to prevent saturation
- π/2 pulse width of 8.4 μs at 18 db power level
- Low power needed for 90% H_2O/ 10% D_2O (75 db) saturation





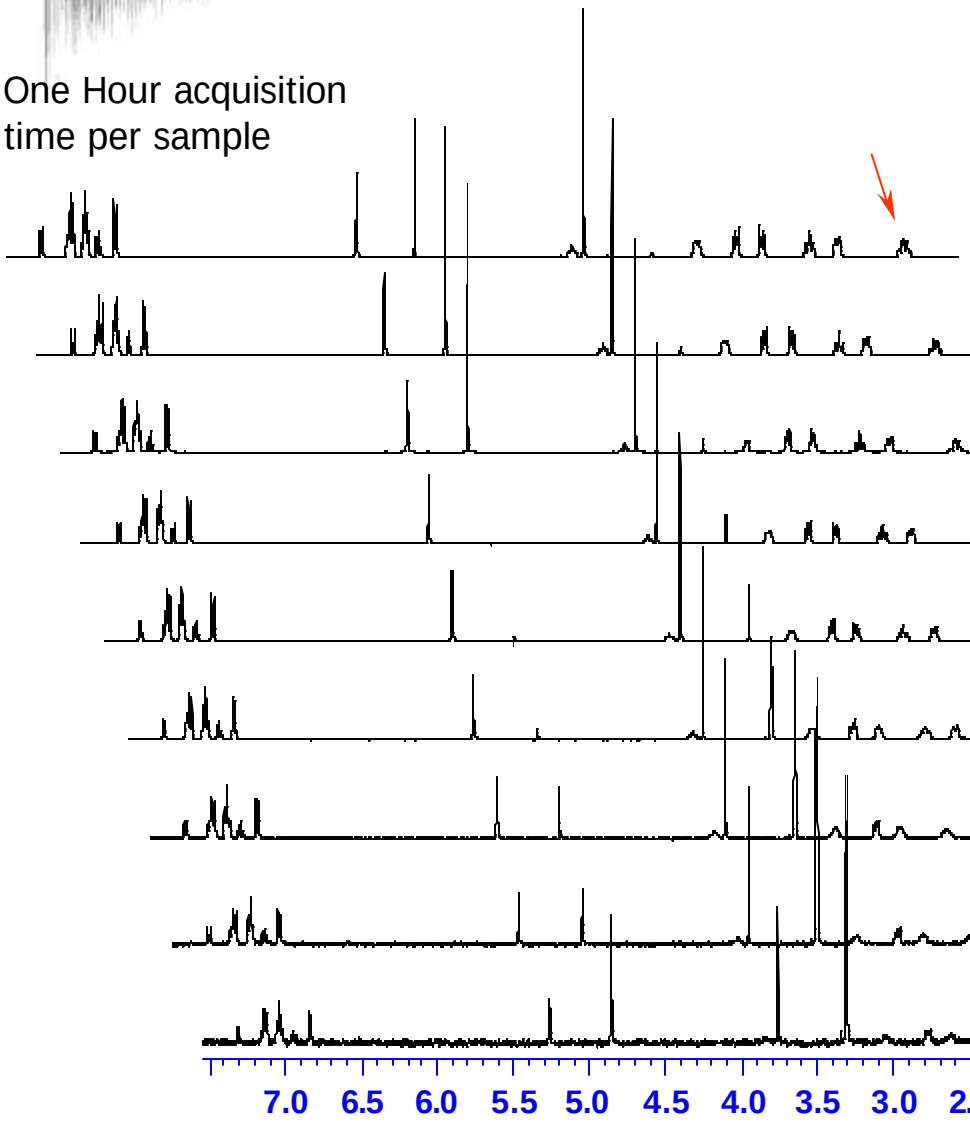
Advantages vs. Disadvantages

- **Advantages**
  - Extremely mass sensitive
  - Capillary-scale fluidics allow transport of µL volume samples over distances of 5-10 meters with virtually no degradation in analyte peak volume.
  - Diffusion and mixing effects at the capillary scale are very limited so that peaks can be parked overnight with negligible loss of S/N.
  - Residual protonated solvents are significantly reduced – need for multiple solvent suppression avoided in most cases.
  - Acquisition of data in fully protonated solvents is reasonable.

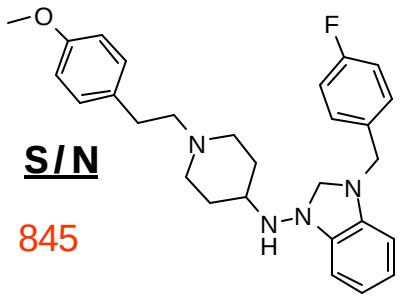
- **Disadvantages**
  - Manipulation of 5µL aliquots can tedious.
  - Availability of HT platform poor
  - Samples of poor solubility

Capillary Probe Data

One Hour acquisition time per sample



Sample in 5mL cell (conc. mg/mL)	S/N
100µg (20 mg/mL)	845
50µg (10 mg/mL)	440
25µg (5 mg/mL)	229
12.5µg (2.5 mg/mL)	116
6.3µg (1.3 mg/mL)	62
3.1µg (0.63 mg/mL)	36
1.5µg (0.31 mg/mL)	25
780ng (0.16 mg/mL)	18
390ng (0.08 mg/mL)	16



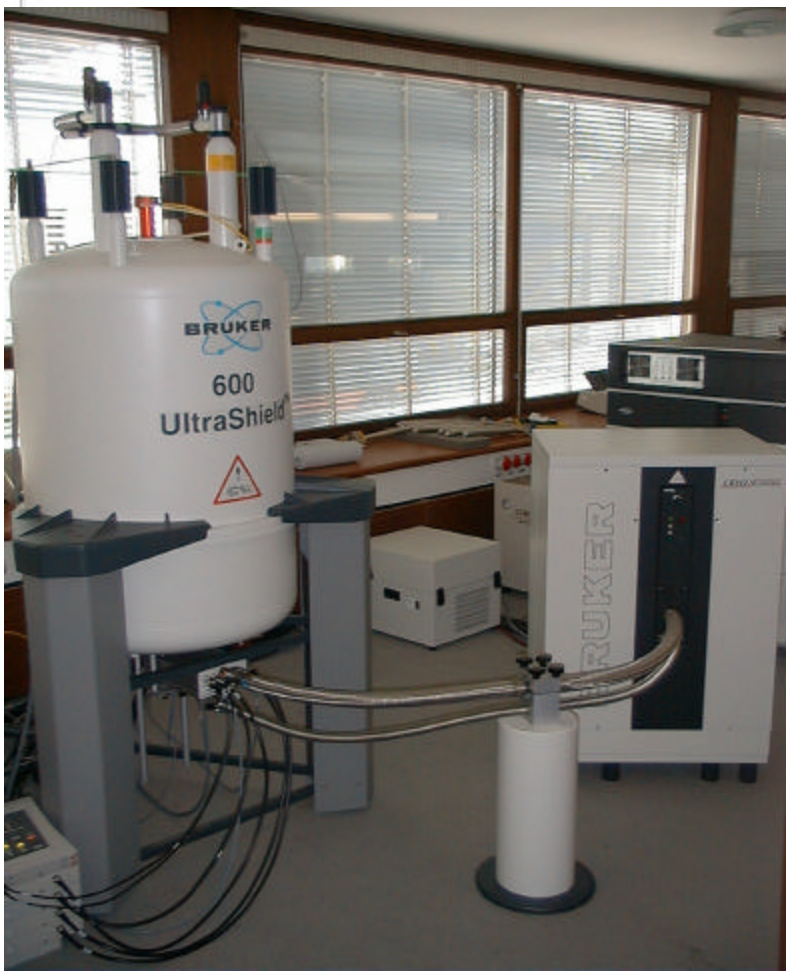
HOD Saturation

Bruker's New MicroTOF -- Metabolites

- The microTOF-LC can provide the exact mass of the analyzed sample and therefore access to the sum formula.
- microTOF-LC allows HyStar™ to trigger collection of chromatographic peaks into loops (LC-NMR), or SPE cartridges (LC-SPE™ NMR) based on mass chromatograms
- However, no additional structural information is provided (e.g. fragmentation). This information is sometimes more relevant than the molecular formula.

Picture taken from www.bruker-biospin.con

CryoProbes



Installation of a 600MHz triple resonance $^{1}H\{^{13}C,^{15}N\}$ CryoProbe™ system.

Picture taken from presentation made by Dr. Kim Colson at Bruker Biospin

Overall Conclusions

- LC-NMR is an extremely useful tool in very specific instances.
- Additional “hyphenation”, in some cases, provides an enormous amount of pertinent structural information.
- Decreasing the noise floor (cryoprobes) is allowing NMR to routinely analyze samples that were previously impossible by NMR

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