

Fast Analytical Quality Preparative UV, ELSD and/or MS Directed Purification for 100 mg Injections

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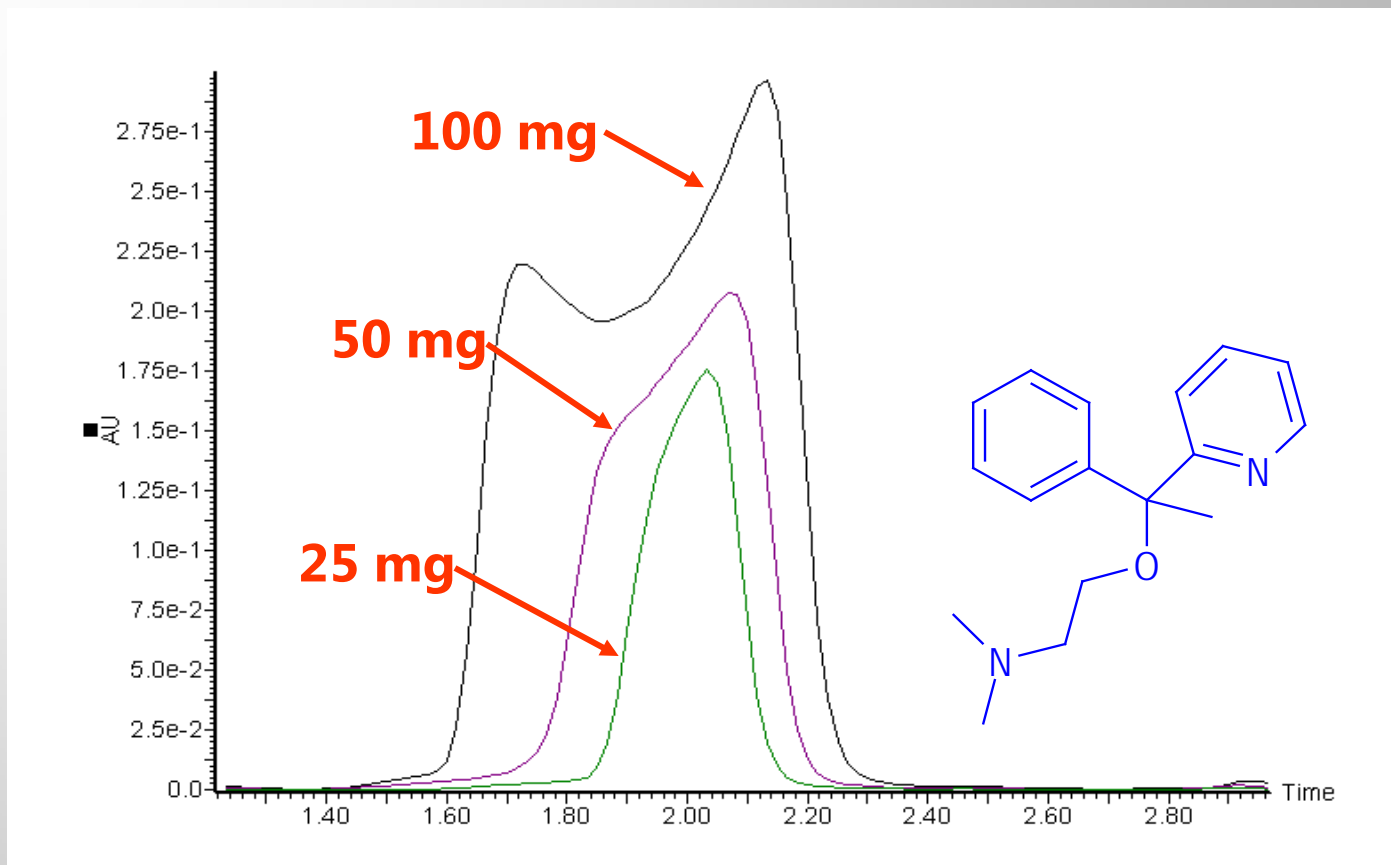
Purification Goals

- ★ **Early Drug Discovery → Full coverage**
 - ★ **Hit to Lead Parallel Synthesis**
 - ★ 20 mg
 - ★ approximately 100 compound / batch every other or third day
 - ★ **Lead Optimization – hand crafted for *in vivo***
 - ★ 100 mg & occasional 0.5 g ultra high purity
 - ★ approximately 10+ compound / batch daily
 - ★ **Development Candidate Candidates**
 - ★ Up to 25 g with at least 5 g at ultra high purity
 - ★ 2 day turnaround on 10 - 15 compounds / yr
 - ★ **Above needs define scale!**
 - ★ Success requires following minimums:
 - ★ 100 mg per injection
 - ★ 1 – 100 compounds / day
 - ★ 125 injections & collections / day

Purification Scale

- ★ **100 mg / injection creates challenges!**
 - ★ **Injection mass increased 10^5 fold but mobile phase volume for separation can increase only perhaps 50 fold compared to analytical scale**
 - ★ Maintaining speed and resolution requires compensation in the chromatographic system.
 - ★ Injection process must be adapted for high load.
 - ★ Adsorption and buffering capacity must be adjusted.
 - ★ **Collection volume and separation time can limit number of compounds collected**
 - ★ High separation efficiency (analytical like) must be routine to keep collection volumes reasonable.
 - ★ High separation efficiency (fast analytical like) must be routine to keep cycle times reasonable.
 - ★ **Additional processes must be automated**
 - ★ Adapted injection process must be automated.
 - ★ Extra adsorption / buffering capacity must be on-line.
 - ★ Automatic column switching & regeneration.

Challenges of Scale



Conventional Wisdom:
Higher mass loading kills chromatographic performance!
Example: doxylamine (pK_a 8.7): 25-100 mg injection. Mobile phase temperature 45°C. Buffer concentration 0.2% formate. Mobile phase pH = 6.5

Challenges of Scale

- ★ **Making 100 mg / injections work!**
 - ★ **Injection via “at column dilution”**
 - ★ Deliver sample fully dissolved to column.
 - ★ Completely separate subsystem (pump, valves).
 - ★ Method adaptable to needs of sample & separation.
 - ★ **Capacity of separation must be high enough**
 - ★ Enough stationary phase surface area to adsorb all sample → 30 cm diameter and temperature control.
 - ★ Higher concentration of sample also can require higher buffer capacity (buffer concentration).
 - ★ Completely separate buffer mixing subsystem.
 - ★ Method adaptable to needs of sample & separation.
 - ★ **Separation efficiency must be high enough**
 - ★ Crucial for speed and purity.
 - ★ Operation in “infinite diameter” mode, i.e. sample not reaching column wall → 30 cm diameter.
 - ★ All other known technique applies, i.e. plumbing, small particles (3 μm), and temperature control.

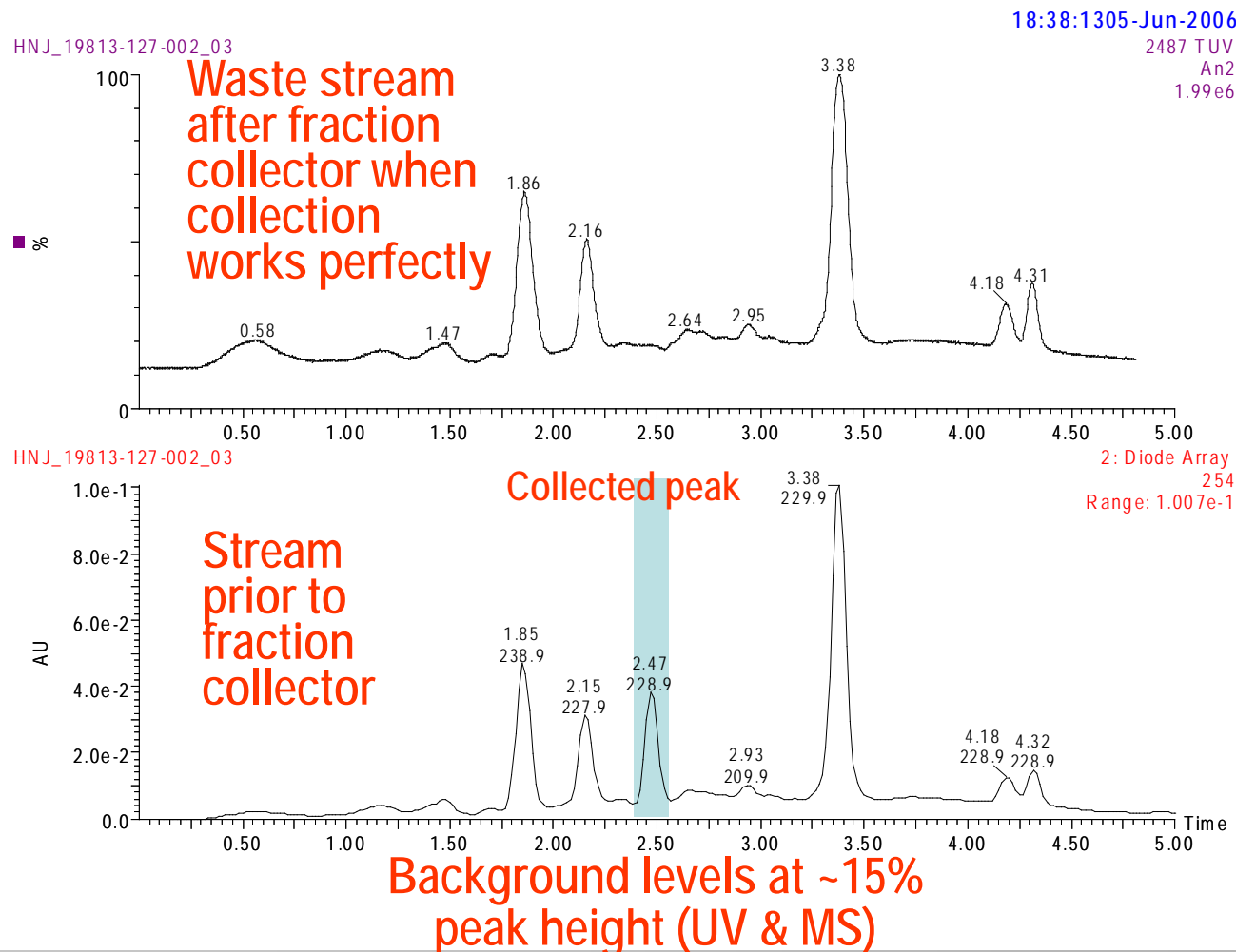
Purification Operational Philosophy

- ☀ Basic tried and true technique: get a quality separation at analytical scale, then scale up (50 fold).
- ☀ 2 x 2 x 2 matrix of gradient LC methods in analytical and preparative scale:
 - ☀ 2 columns – C_{18} and C_8 .
 - ☀ 2 gradients – C_{18} gradients favor polar compounds and C_8 non-polar.
 - ☀ 2 pHs – 4 and 7.5 (extremes rarely needed).
- ☀ Routine 100 mg per injection & Gaussian peaks.
- ☀ Success rate as close to 100% as possible (measure and collect waste, if needed).
- ☀ Must be automated with optimized conditions built into predictable, calibrated methods.
- ☀ The following setup is being routinely utilized.

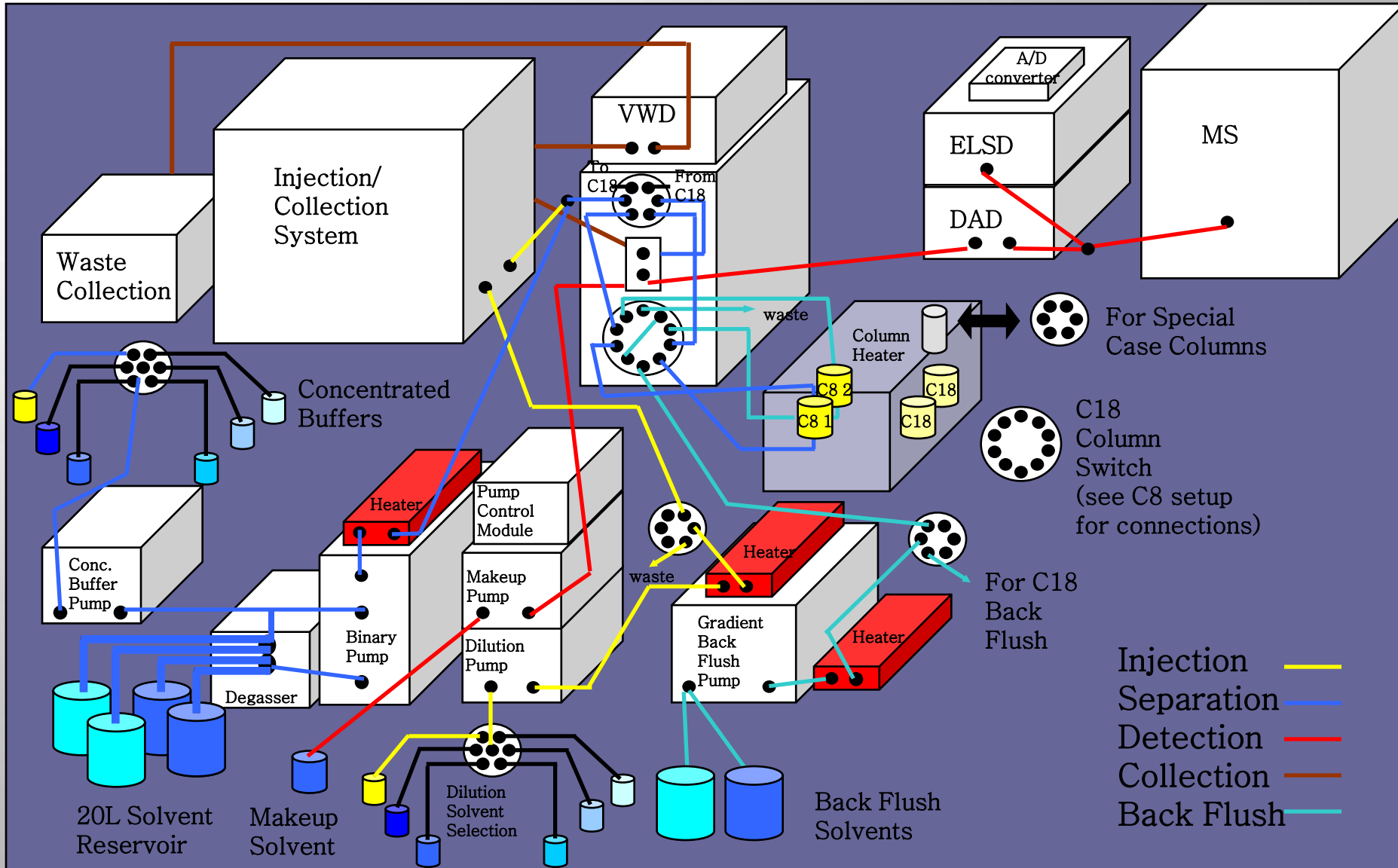
Operational Philosophy: Why use analytical data and scale up?

In many cases, only data from the actual sample allows accurate threshold prediction due to earlier eluting peaks and baseline rise from low level impurities.

Losing compounds is not a viable option for achieving increased speed.



A Fully Automated Purification Setup



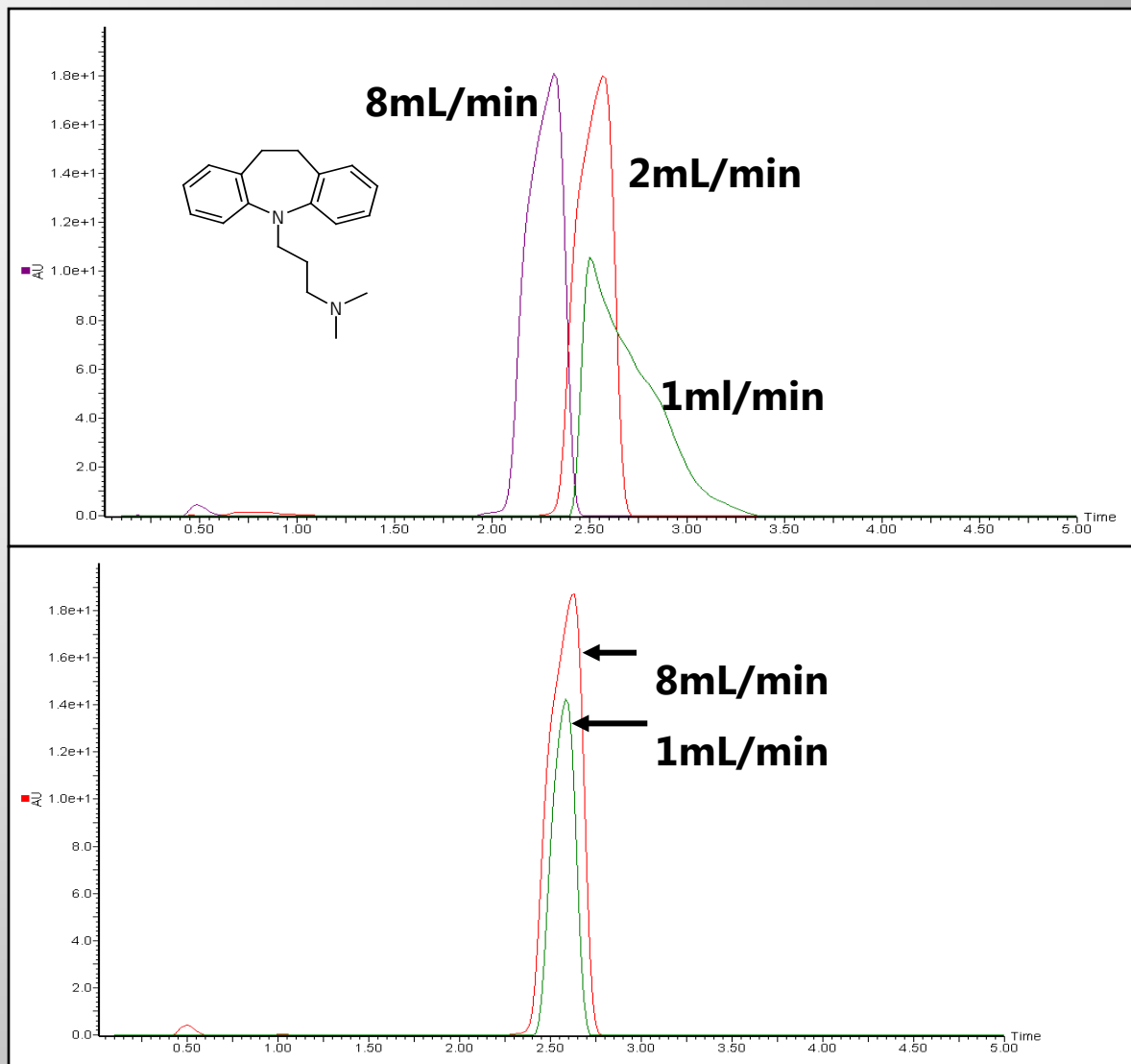
"At Column Dilution" Approach for Sample Injection

- ☀ **Goal: Deliver sample to stationary phase as individual molecules in solution.**
- ☀ Flow parameters are important.
 - ☀ Flow rate must be sufficiently high to deliver sample without wasting time (7.5 mL/min into 100 mL/min).
 - ☀ Diverting at column dilution solvent after injection process is important to eliminate the effects of injection solvent on the separation (divert at 0.5 min).
- ☀ Injection solvent composition is an untapped resource for scaling up injection mass.
 - ☀ Choice of injection solvent can have big impact on keeping samples in solution as injection mass increases and can help chromatographic performance.

At Column Dilution – Flow Rate & Divert

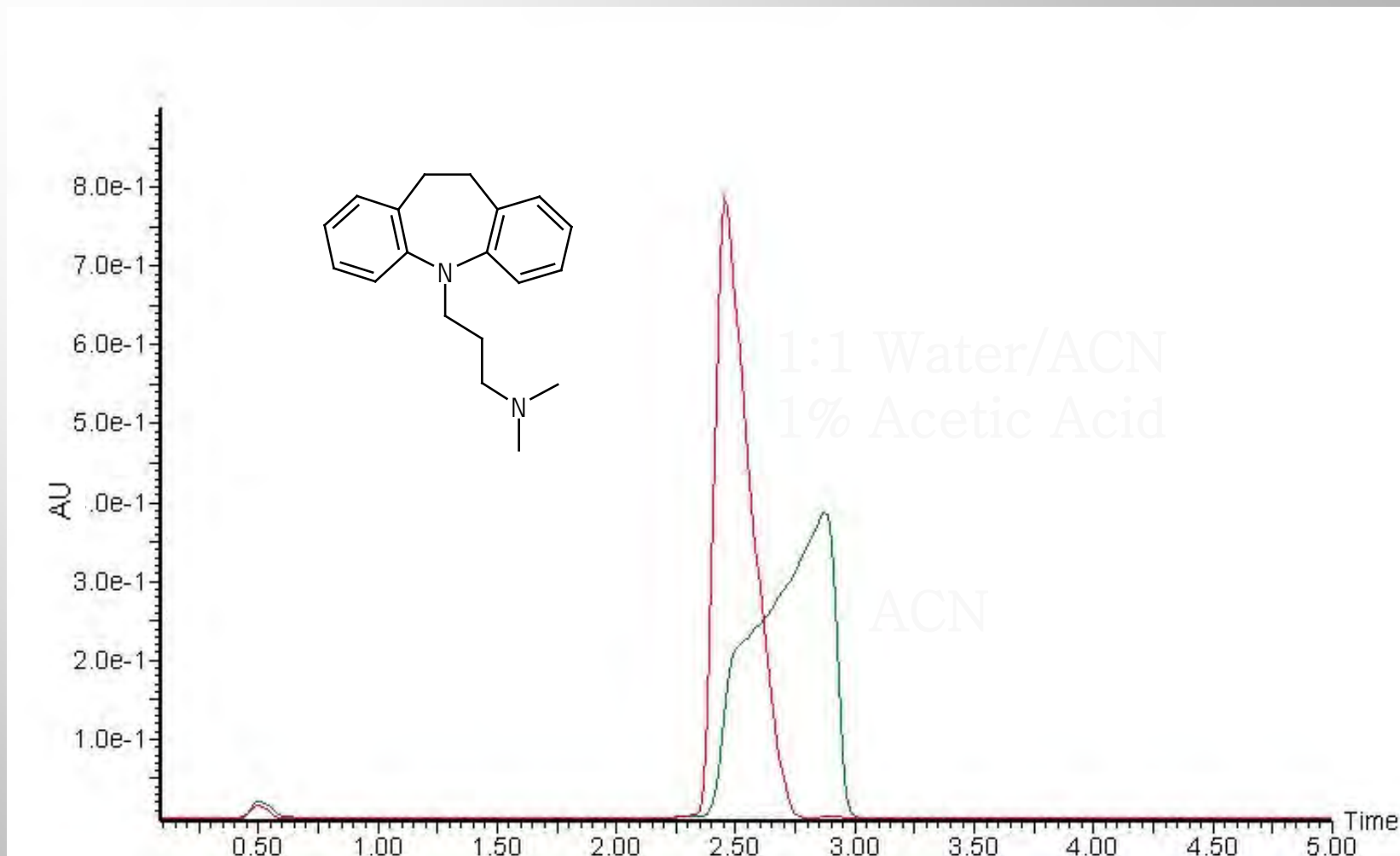
Example: effect on imipramine injection & separation

- Elevated flow rate to avoid wasting time.
- Target: injector sweep time 15 s or less.
- Diverting “at column” flow also can improve separation by avoiding buffer capacity reduction.
- Elevated flow rate also helps prevent sample loss.



At Column Dilution - Solvent Choice

Example: effect on imipramine injection & separation



Change in solubility by lowering pH eliminates injection precipitation.

Stationary Phase Capacity

How to address it...

☀ Column Choice:

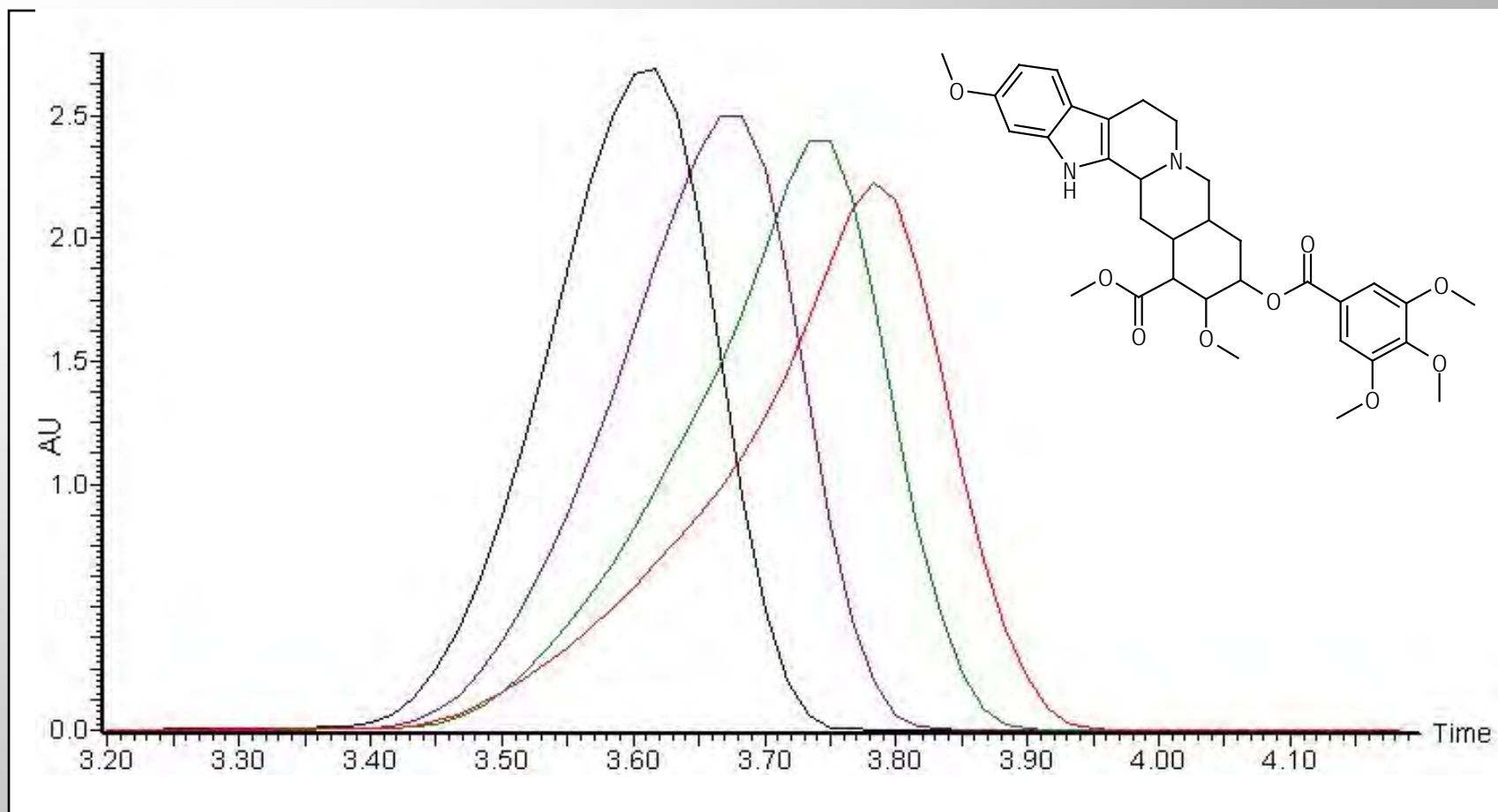
- ☀ Smaller particles (3 μm) enhance surface area and adsorption capacity.
- ☀ 30 mm diameter provides enough capacity for 100 mg injections.
- ☀ Smaller particles (3 μm) increase separation efficiency and speed.

☀ Temperature:

- ☀ A crucial parameter that affects adsorption and thus must be properly controlled:
 - ☀ Temperature related to adsorption rate by Arrhenius equation.
- ☀ Four independent heaters used:
 - ☀ Mobile phase heater (400 Watts)
 - ☀ Column heater (water bath maintained at temp of mobile phase)
 - ☀ Dilution solvent heater (10 Watts).
 - ☀ Back-flush solvent heater (400 Watts)
- ☀ Benefits of Temperature Control:
 - ☀ Improved peak shapes due to more adsorptions (fronting = missed adsorption opportunities).
 - ☀ Reduces back pressure allowing higher flow rates and faster runs.
 - ☀ Maintains highly concentrated samples in solution. This is especially crucial during the injection process.

Temperature Effect on Peak Shape

Reserpine



Mobile phase and column heaters were maintained at the temperatures indicated to achieve the improvement in peak shape observed.

Mobile Phase Buffer Capacity

How to address it...

☀ Background:

- ☀ Goal: send desired compound through column un-ionized.
- ☀ Buffer must be more concentrated than analytical scale because sample is more concentrated.
- ☀ Basically follows Henderson – Hasselbach equation and thus pH and pK_a are equally important.
- ☀ KEY MESSAGE: must view un-ionized state loosely as ion pairing with buffer can be sufficiently un-ionized for good separation peak shape for bases where $pH < pK_a$ ($pH - pK_a = -1$ or -2 is OK!).

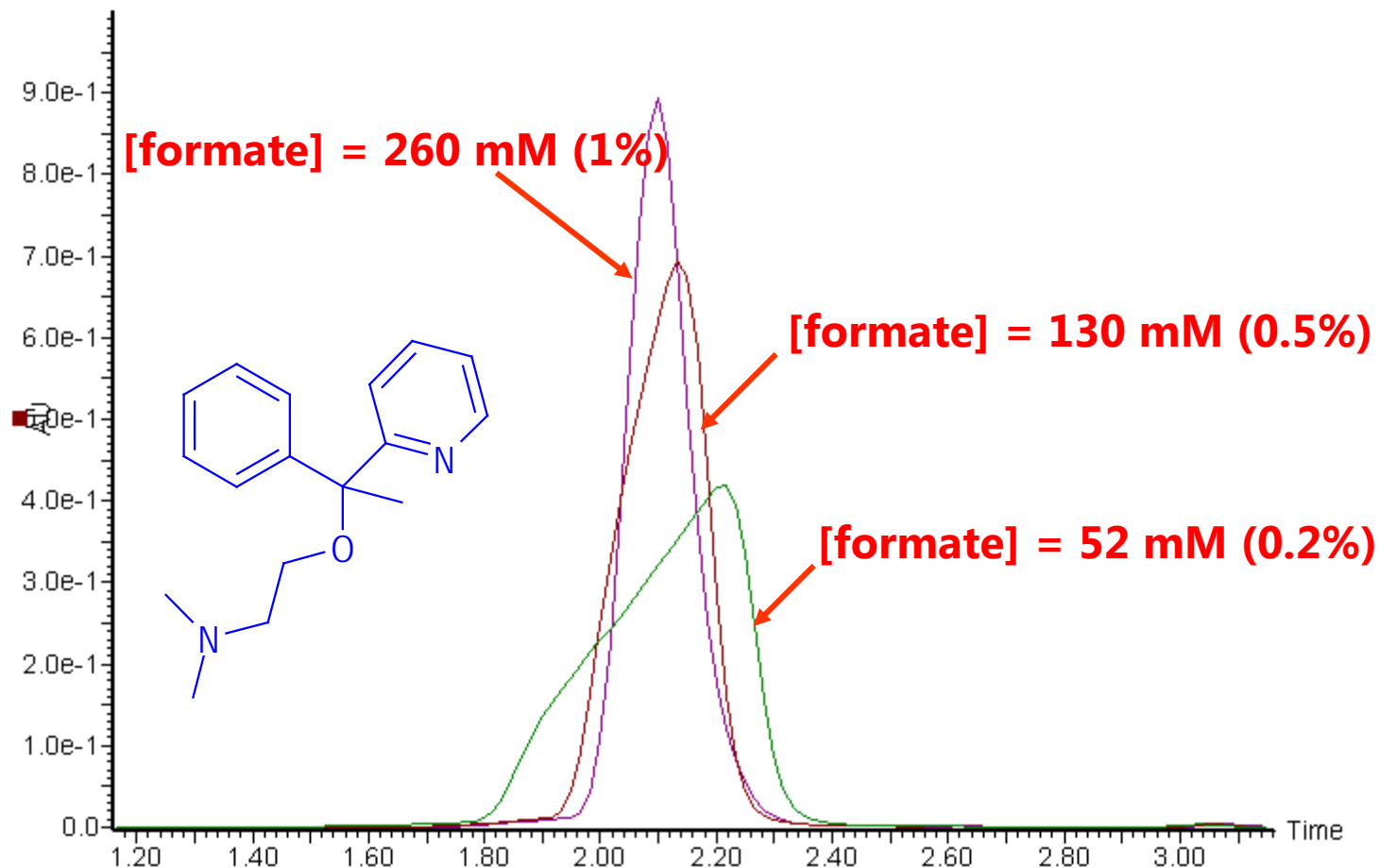
☀ Practical Approach:

- ☀ Mix buffer on-line like “at column dilution:”
 - ☀ Flow rate proportional to buffer concentration.
 - ☀ Valve makes it easy to have 6 buffers on-line & method selectable.
- ☀ Target high pH and buffer concentration for high pK_a compounds:
 - ☀ pH 4 and 7.5 can cover a very full range of drug like compounds.
 - ☀ Lower buffer concentration (0.2%) usually works well for bases when $pH > pK_a$. Also, more quickly removed during drying process.
 - ☀ Higher buffer concentration crucial for high mass loading.
 - ☀ Higher buffer concentration (1%) at pH 7.5 usually works well with high loading of bases when $pH \leq pK_a$.

Mobile Phase Buffer Capacity

Effect on peak shape & efficiency

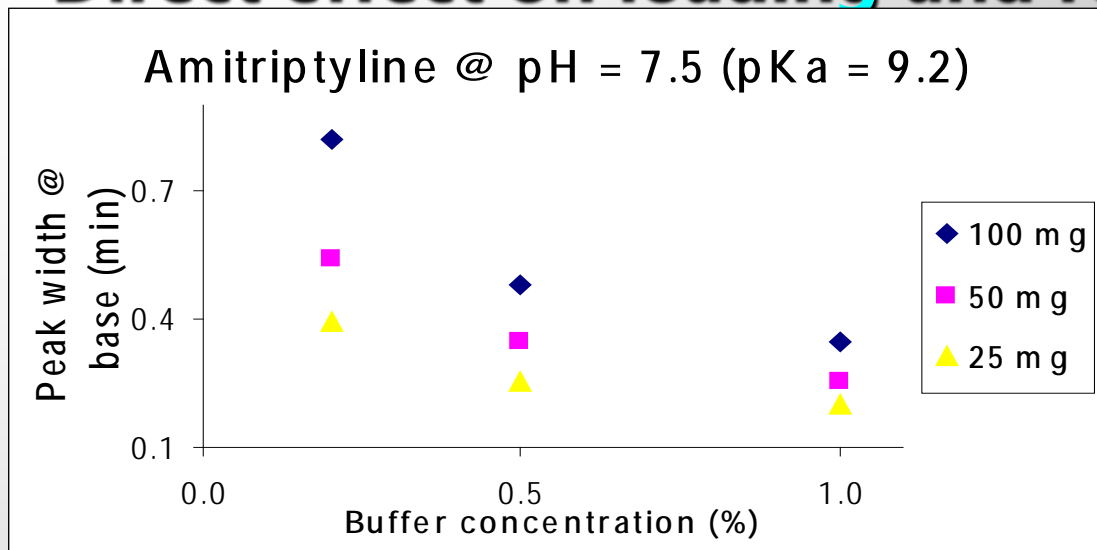
Doxylamine
 $pK_a = 8.7$



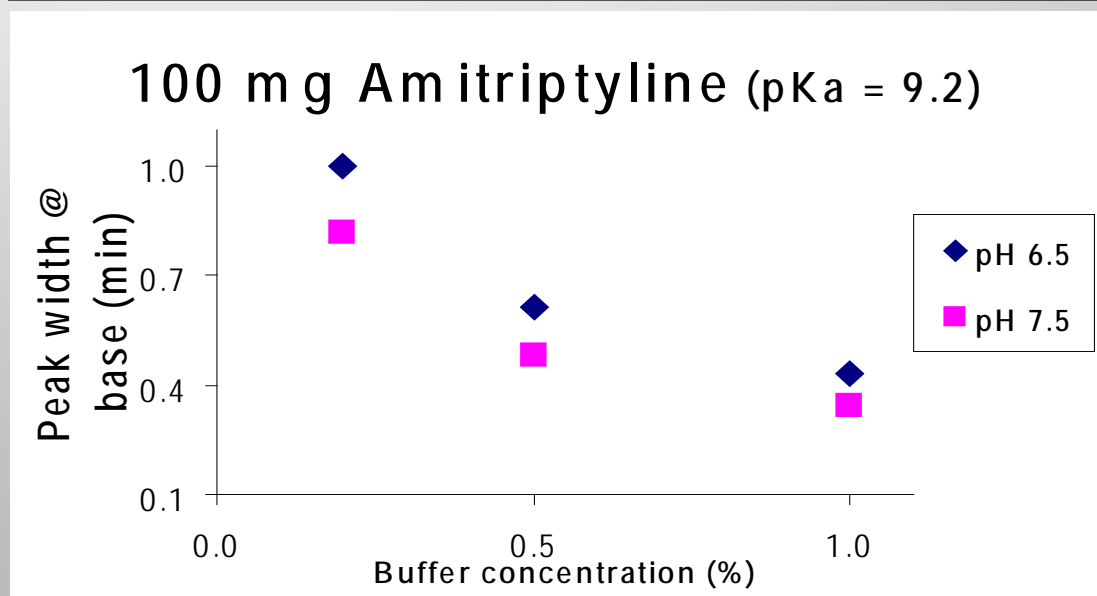
100 mg injection. Mobile phase pH = 7.5
Mobile phase temperature 45°C.

Mobile Phase Buffer Capacity

Direct effect on loading and relative to pH shift



Buffer capacity has a large impact on column loading (peak width).



Note the buffer capacity effect is considerably larger than the pH effect on loading.

Column Back-flush Regeneration

A simple but crucial component for success

☀ **Back-flushing technique**

- ☀ Gradient back-flush repeated 3x over duration of run
- ☀ Flow rate of 20mL/min sufficient (1/5th)
- ☀ Acidic buffer (5% acetic acid in water) and organic (1% acetic acid in ACN) phases used.

☀ **Benefits of back-flushing column**

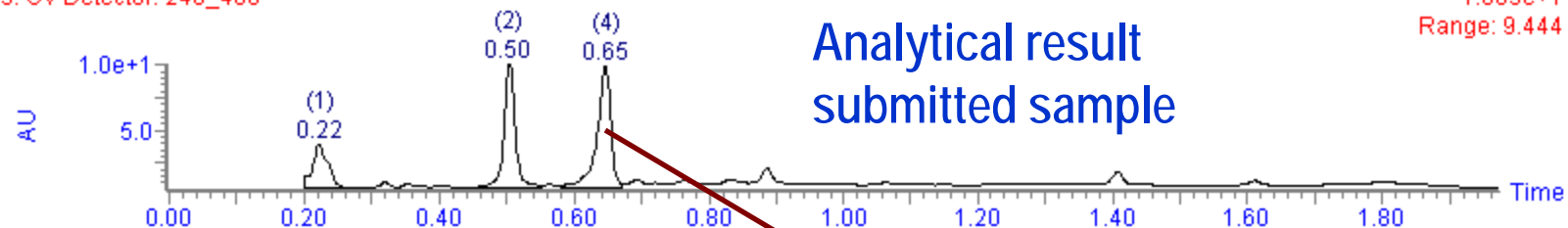
- ☀ Prolong lifetime of expensive prep columns (>10 fold).
- ☀ Prevents carryover.
- ☀ More consistent performance.
- ☀ Allows for re-equilibration of column prior to starting next cycle, i.e. no time lost.

Purification Examples

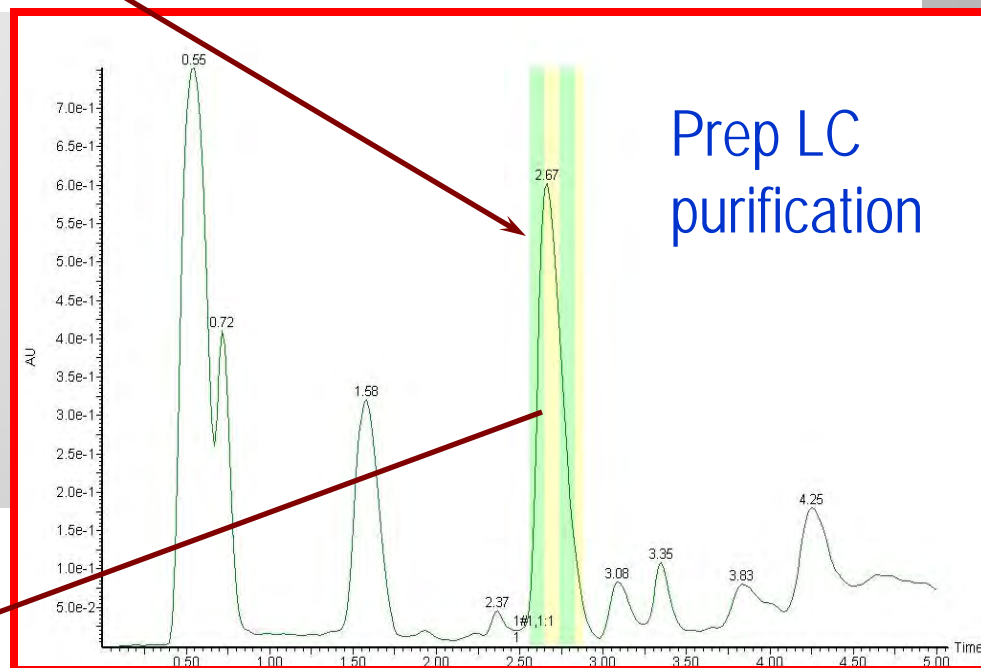
- ☀ Variety of samples purified.
 - ✳ One shot samples (purification 50 - 100mg).
 - ✳ Libraries requiring 100s of injections/collections.
 - ✳ Purification of gram quantities requiring repeated injections of 100mg or more.
- ☀ Variety of sample qualities encountered.
 - ✳ Quantity of sample ranges from 5mg to 25grams.
 - ✳ Sample purities range from 5 to 95% prior to purification.
 - ✳ Impurities may or may not be base line resolved according to OA-LCMS.
 - ✳ Dissolution remains an ongoing challenge.

Purification of Synthesized Product

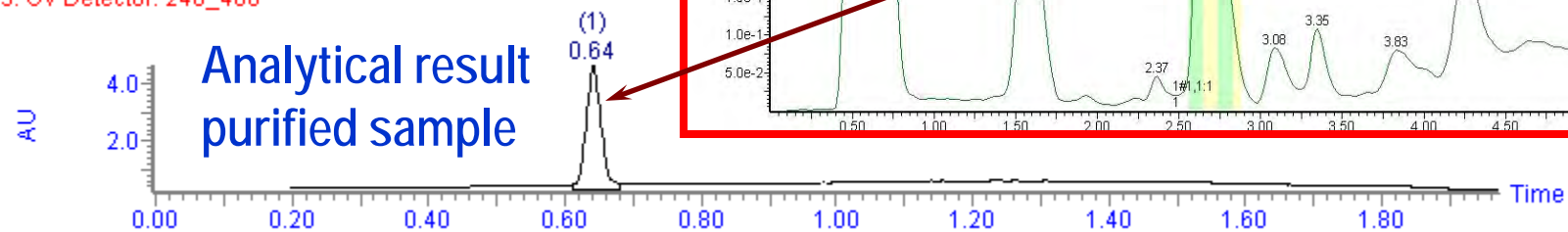
3: UV Detector: 240_400



Purification performed using a neutral pH method and C₁₈ column
Temperatures of 40°C used



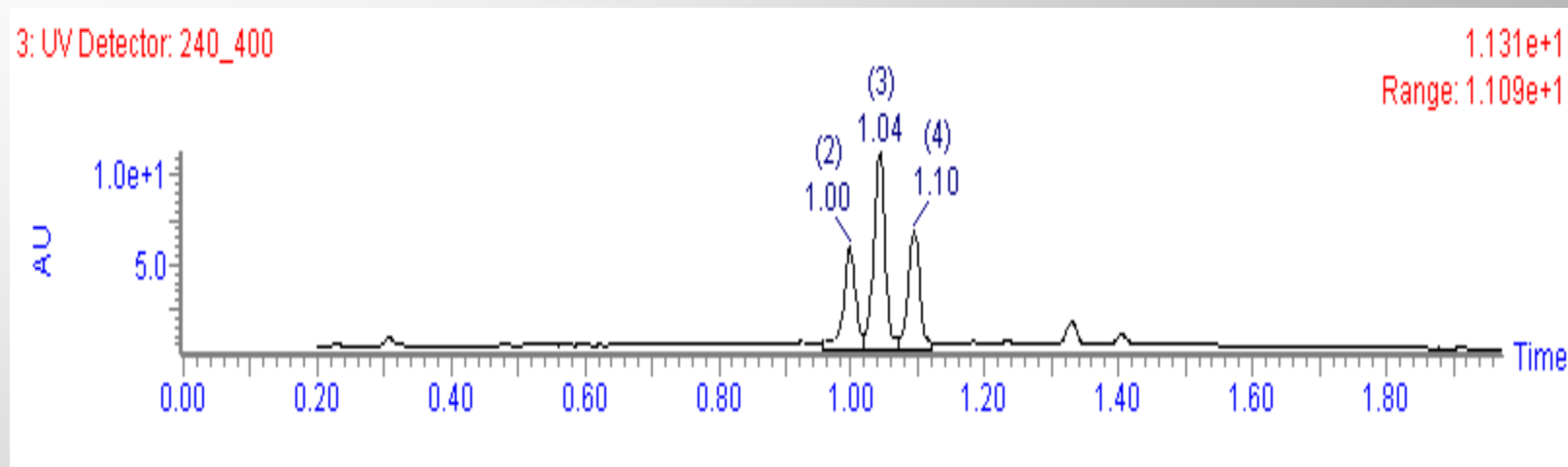
3: UV Detector: 240_400



Purification of Closely Eluting Species

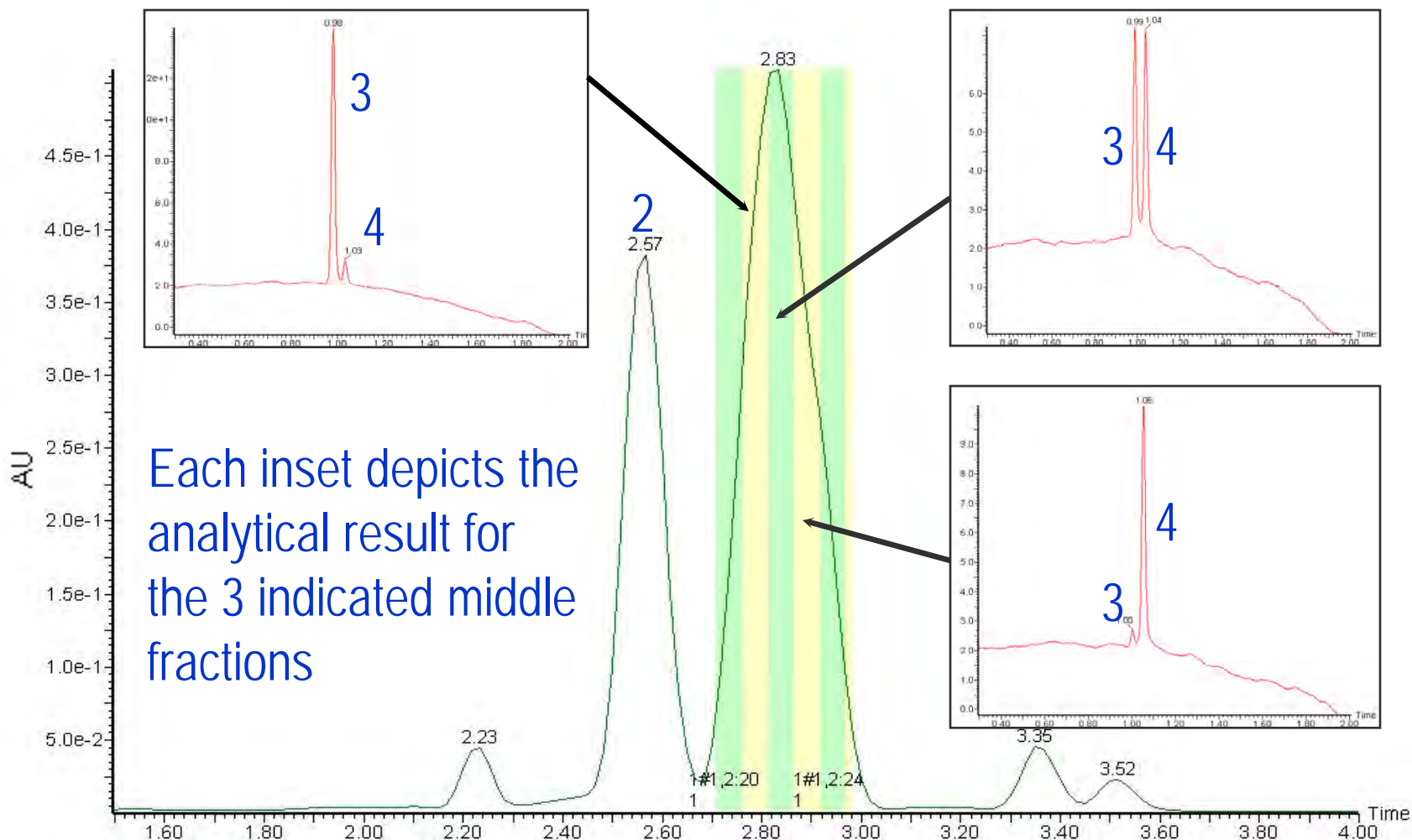
Diastereomers 3 and 4

Analytical Result for Mixture of Diastereomers



Isolation of both diastereomers (peaks 3 and 4) required.
Isocratic method utilized to maximize resolution of two diastereomers.

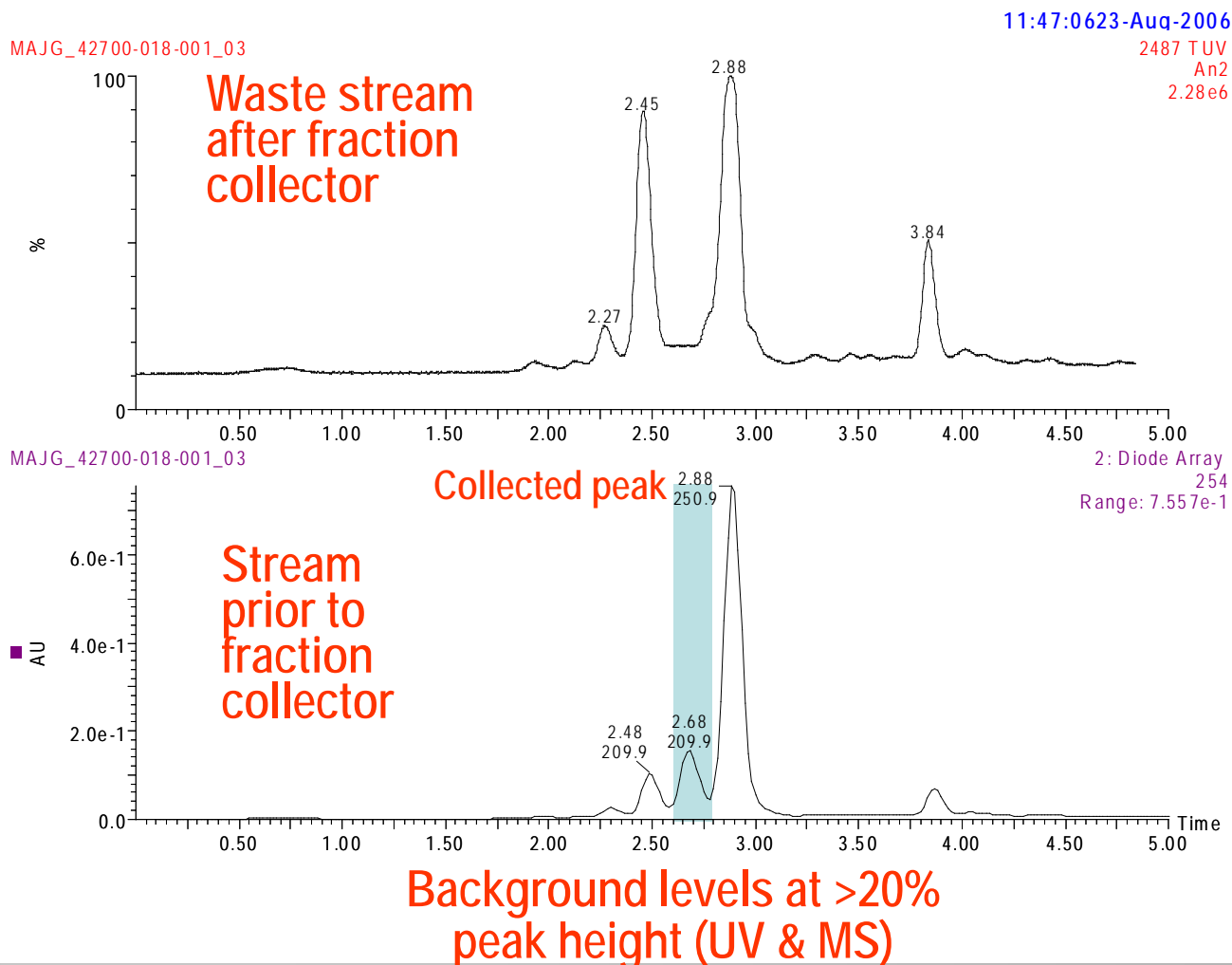
Separation of Diastereomers from crude mixture



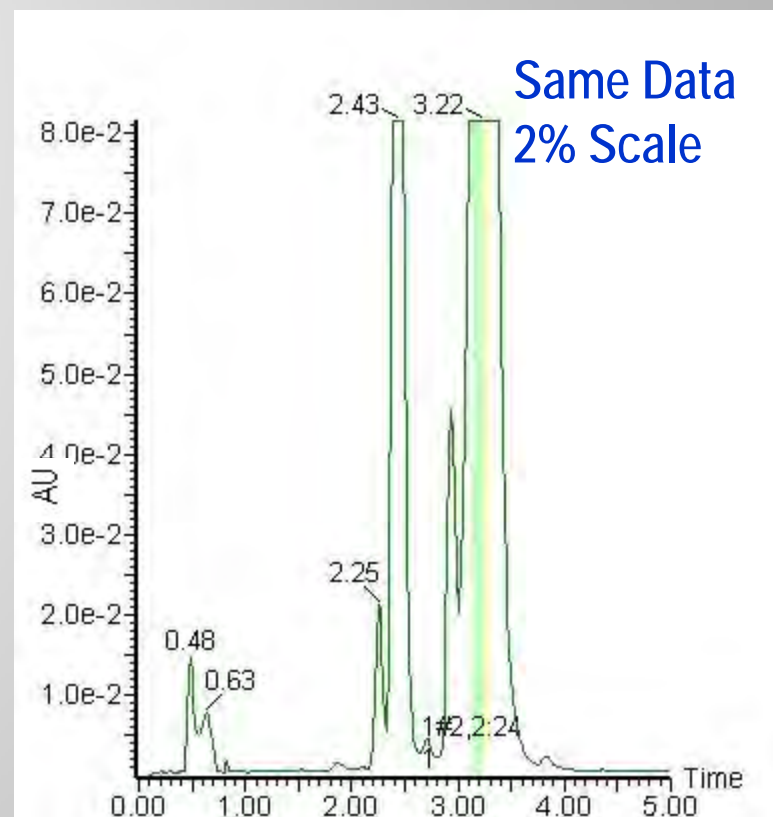
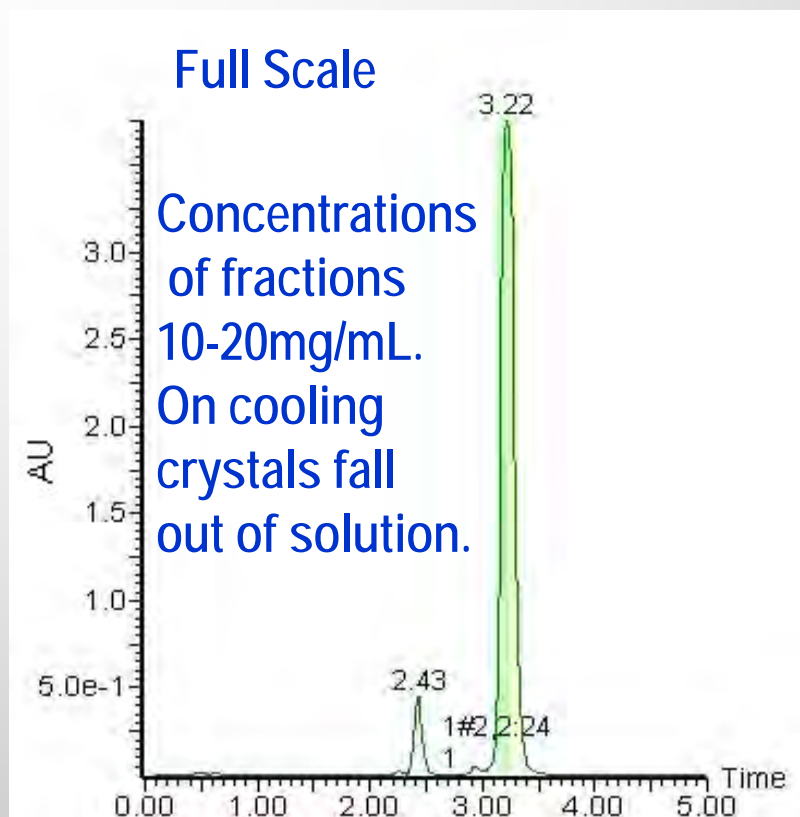
Purities of 95% were achieved for diastereomers 3 and 4.

Purification of Closely Eluting Species with high background

Purities of
>95% are
routinely
achieved for
samples such
as these.



120mg Injection of Drug Candidate



Typical injection for purification of gram quantities of material. In this case 8 grams were purified within 8 hours with a purity of >99.9% (for toxicology study).

Level of recovery was estimated at >90%.

Summary

- ☀ **Achieved analytical quality for 100+ mg injections.**
 - ✳ Good RT / threshold correlation with OA-LC/MS.
 - ✳ Gaussian peaks.
- ☀ **High throughput.**
 - ✳ High velocity separations – 3.5 mm/s.
 - ✳ 5 min run time, $k' = 20$ separations.
 - ✳ Column switching eliminates need for equilibration time.
 - ✳ Theoretical >200 compounds purified per day OR >20 g single compound (multiple injections).
- ☀ **High Reliability/Success Rate.**
 - ✳ >99% success rates with full automation.
 - ✳ Back-flushing prolongs life of columns (>1000 injections per column)
 - ✳ >100 grams purified on each column.
- ☀ **Versatile**
 - ✳ Range of buffers and columns selected (C_8 and C_{18}) cover a wide range of compound purification applications.
 - ✳ Equipped with additional column selection to allow for purification of highly basic compounds (high pH) and if desired reverse phase chiral purification.

Method Summary

Flow Rates: 100mL/min total with 1-5 mL/min buffer and 7.5mL/min from the dilution pump for the 1st 0.5minutes.

Temperatures: 55°C for mobile phase, columns and back-flushing and 70°C for the dilution heater.

Columns: C₁₈ Inertsil ODS-3, 30x50mm, 3 μm particles.

C₈ Inertsil C8-3, 30x50mm, 3 μm particles.

Tubing ID: 0.03" prior to column, 0.02" after column.

Splitter: 1/10000 split with MeOH / 0.1% HCOOH as makeup solvent.

Mobile Phase: Organic, ACN UV grade from B&J

Neutral, 0.2-1.0% ammonium formate in 1%ACN/water

Acidic, 0.2-1.0 % acetic acid in 1% ACN/water.

Dilution Solvents: Varies but predominantly 1:1 ACN/water.

Back-flush Solvent: (A) 5% acetic acid in 1% ACN/water
(B) 1% acetic acid in ACN.