"Theory and Practice of High Speed Chromatography for Bioanalysis"

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General Observations about HPLC Separations

- A good separation is necessary for good bioanalytical quantitation.
  - Resolution from unknown interfering compounds such as metabolites and "isobaric" compounds with same nominal mass but different identity as our analyte.
  - Good separations have traditionally meant a long gradient and equilibration.

How can we speed up our separations?

What is our goal?

More Observations

- As we increase the system temperature, (column oven, etc.) retention time decreases. Peaks my show better symmetry.
- As flow rate increases, peaks become narrower.
- As particle size decreases, resolution improves, but system pressure also increases.

How can we control these parameters?

Equations to help understand what's going on…

Retention, k'

(also called Capacity Factor)

$k' = V_1 - V_0 / V_0$

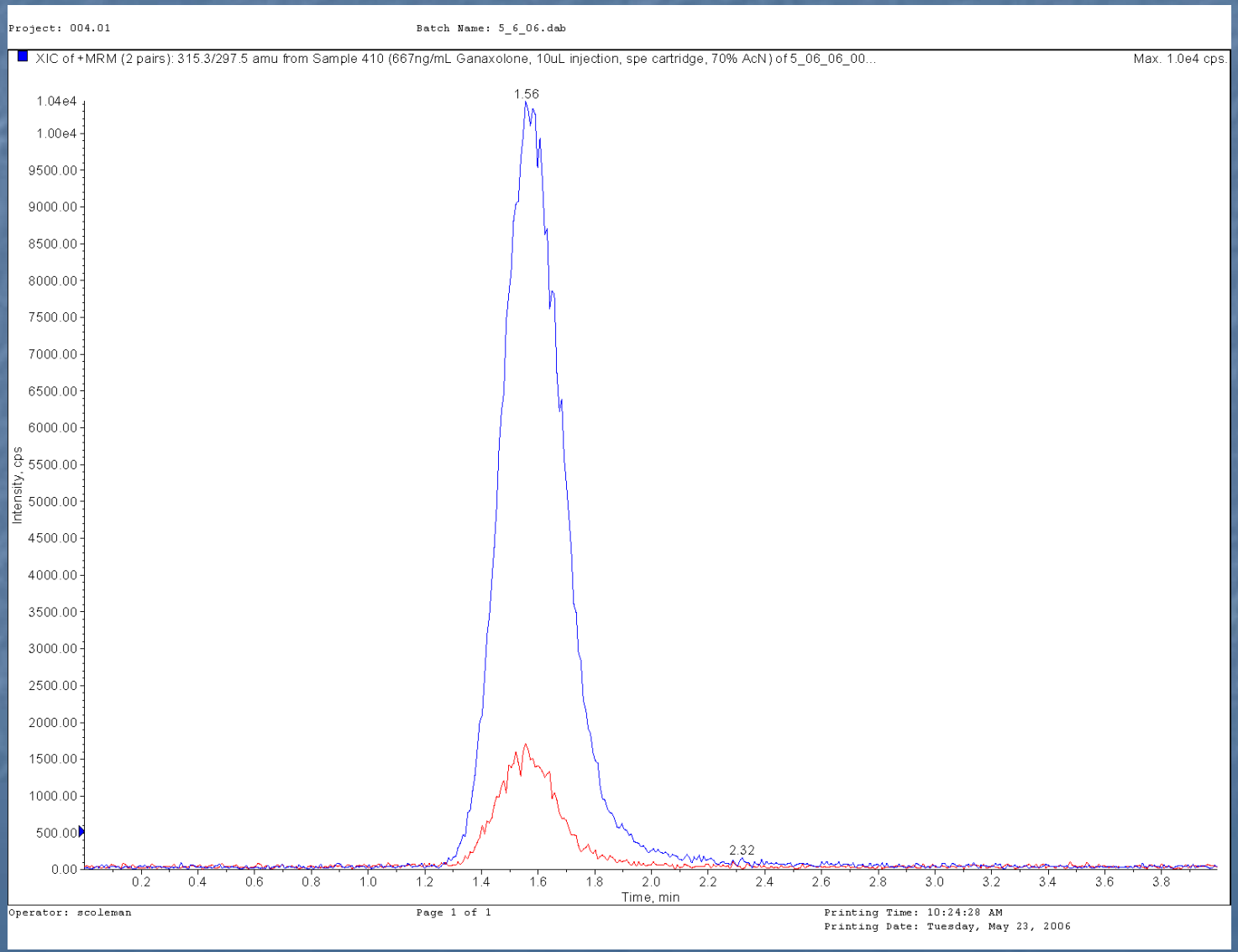
Where V_0 is the volume of an unretained compound and V_1 is the volume of the peak of interest.

k' is a measure of how well a compound is retained by the column & HPLC system.

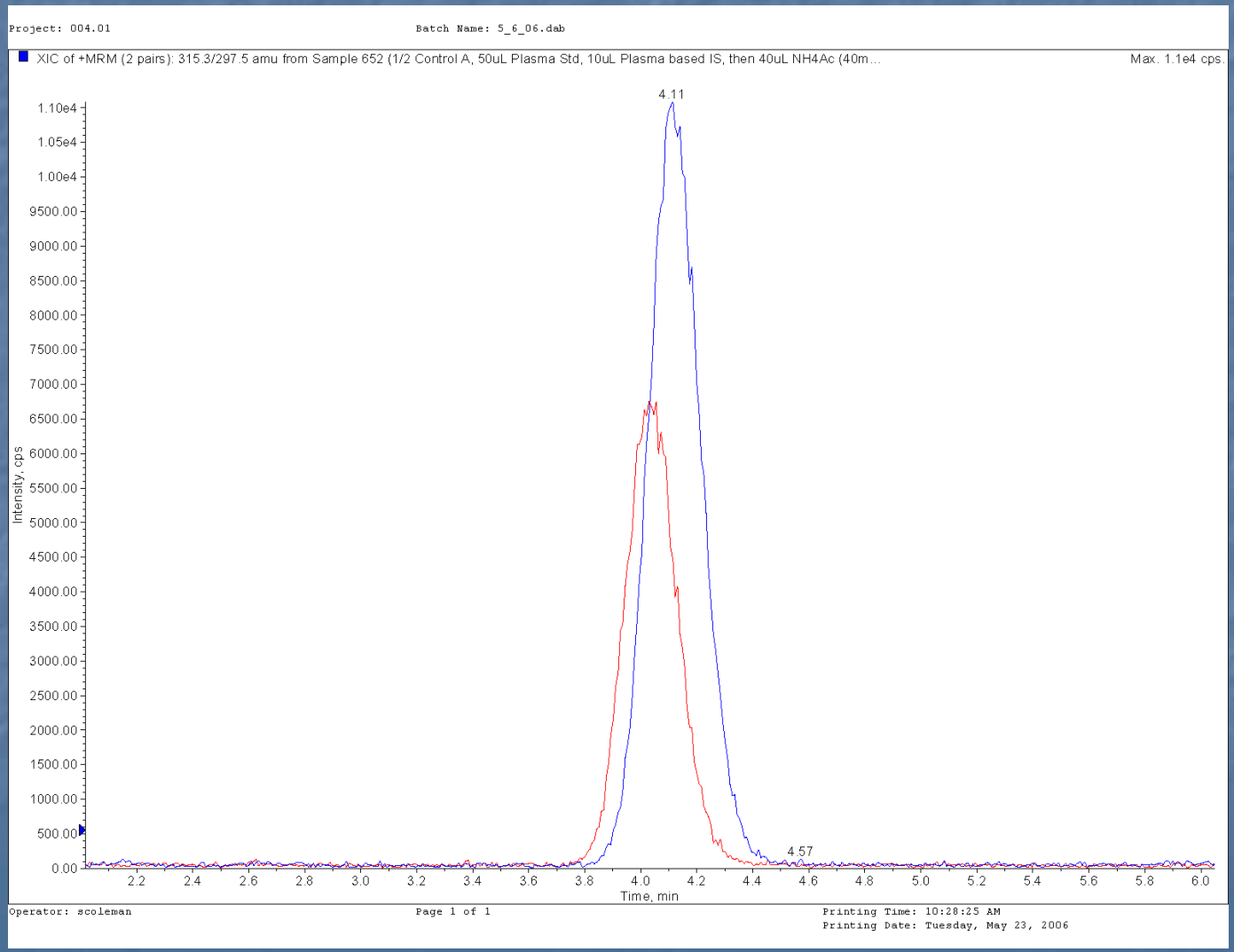
k' is a phisiochemical property of a given substance relative to the stationary phase and the solvent system/gradient.

For a given system/analyte combination, k' is always the same, regardless of solvent velocity.

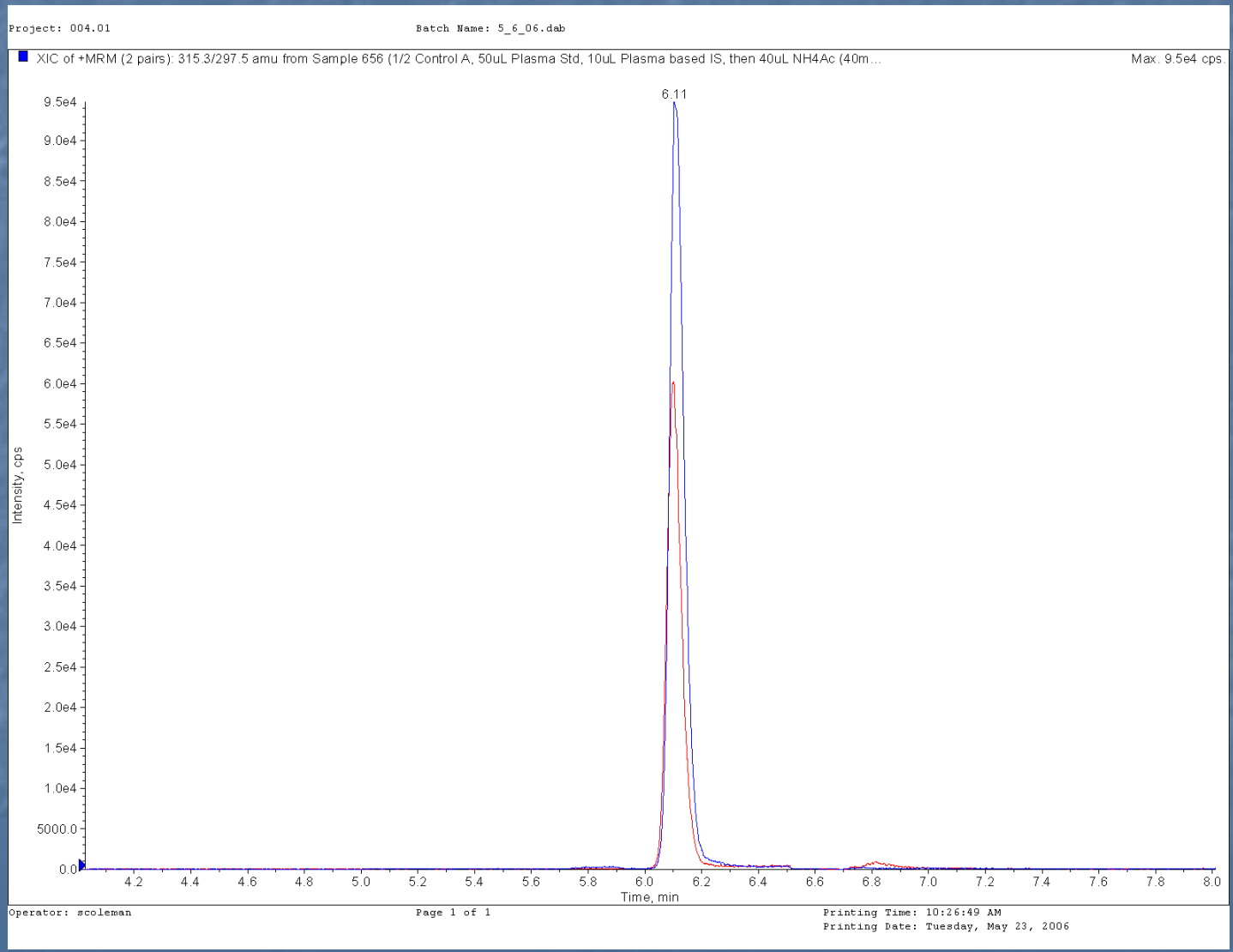
Poorly Retained Compound
("validated", isocratic method provided by client)



Same compound after mobile phase adjustment (% organic lowered). Method still isocratic.



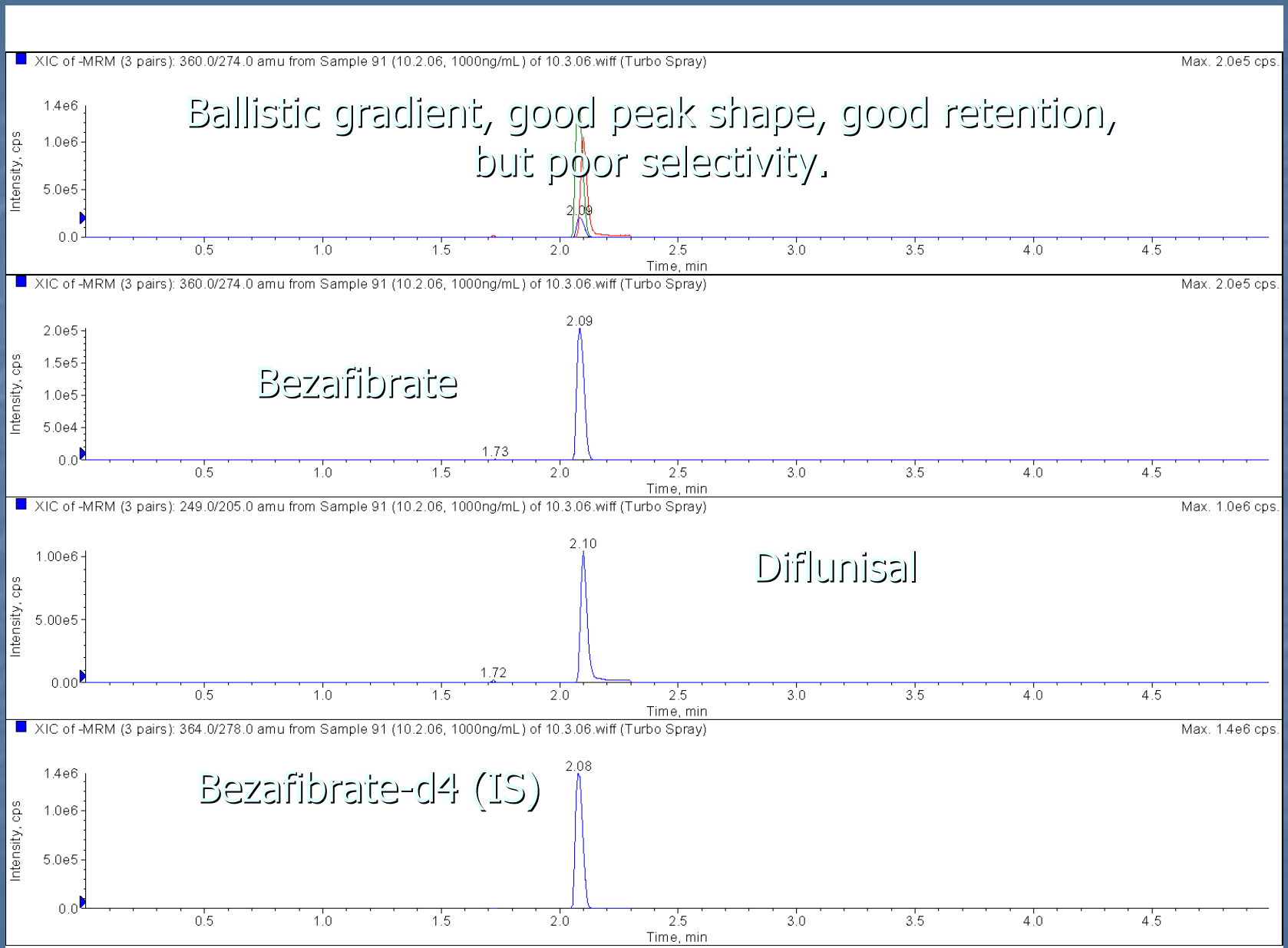
Gradient method. Same compound, but now with excellent retention, better peak shape, and non-interference from Glucuronide metabolites.



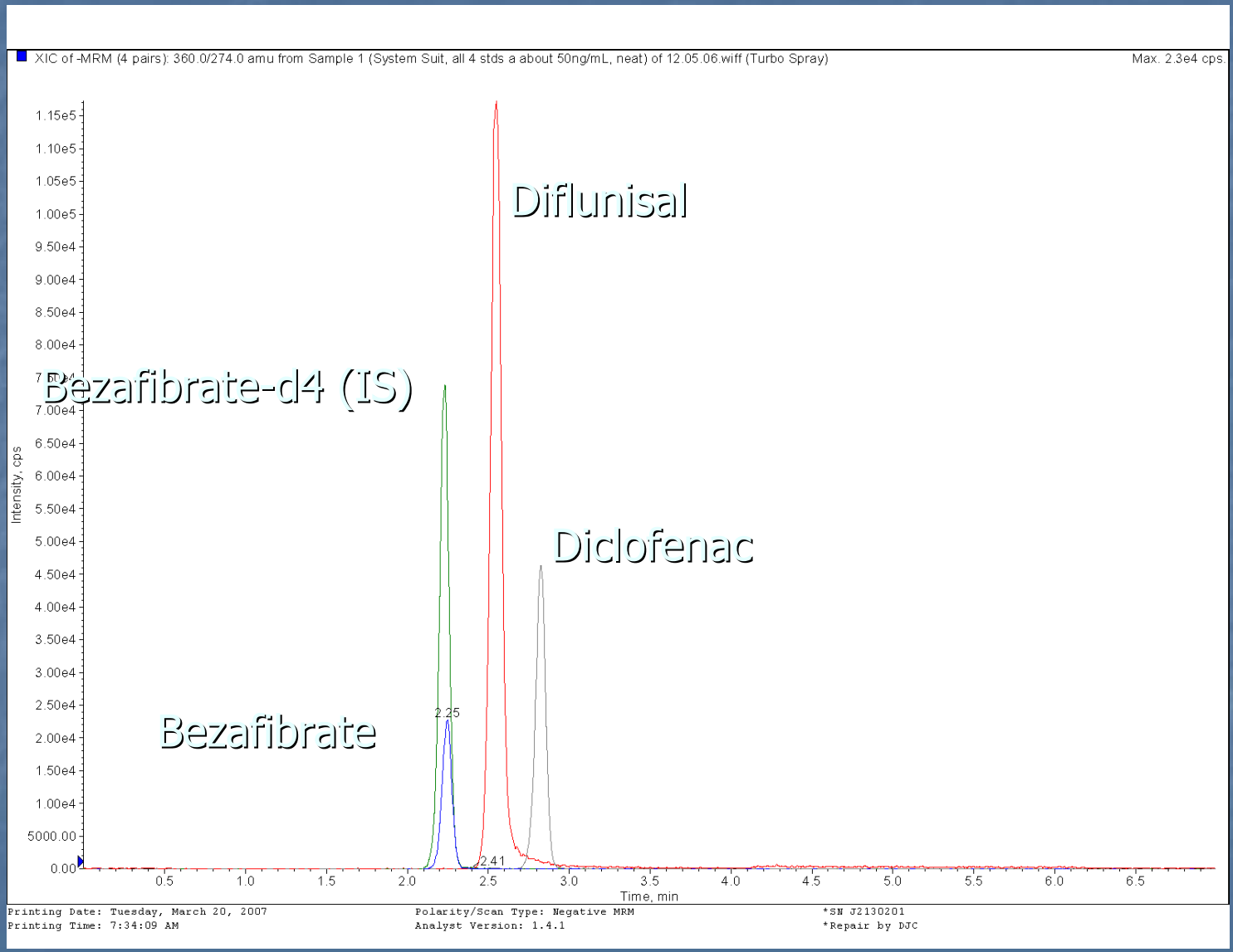
Selectivity, α

- $\alpha = k_2 / k_1$
- A highly selective system will differentiate between two compounds. k_1 will be far apart from k_2

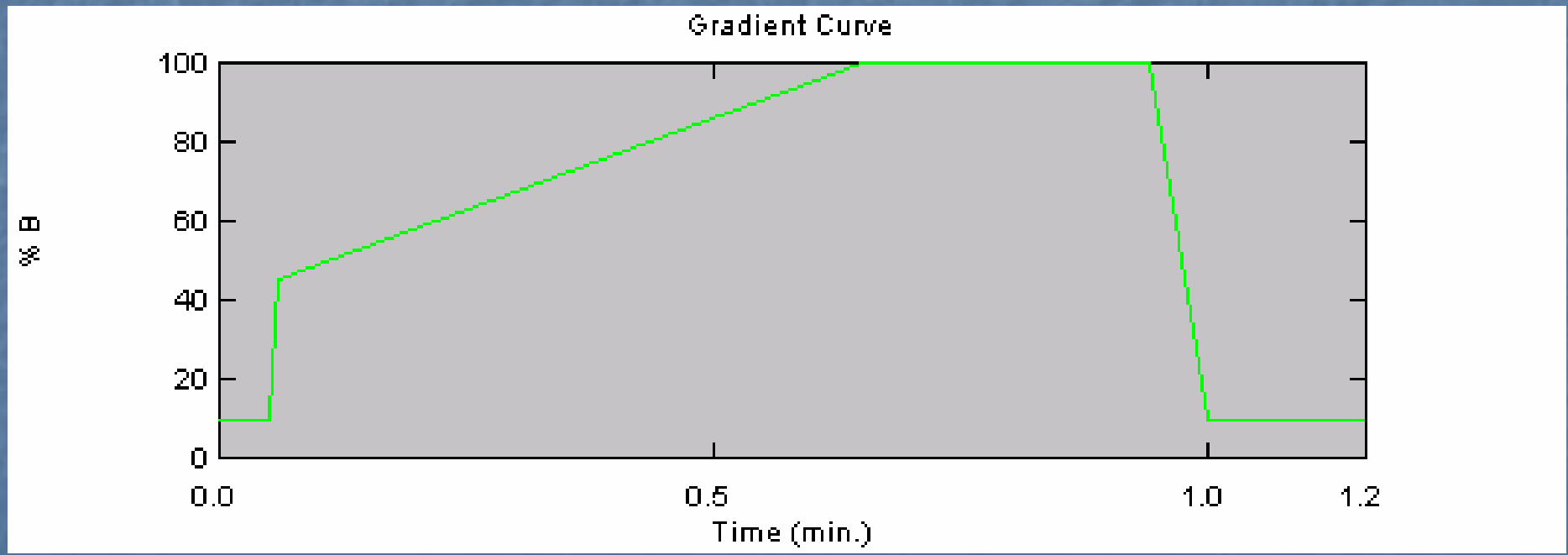
Example of O.K. Retention and poor Selectivity



Example of both good Retention and Selectivity



example of rapid gradient program that may provide both separation and selectivity (depending on analytes)



Separation Efficiency, N

- Separation Efficiency is often defined as N, the number of theoretical plates in a column.
- $N \propto RT$ *(retention time)/ peak width*
- An efficient column will yield narrow peaks even on strongly retained compounds that have long retention times.

Peak Symmetry

- For good quantitative results, symmetrical peaks are important.
- Poorly shaped peaks, (broad, split, fronting or tailing) result in lack of precision and accuracy and in poor detection limits.

Factors affecting peak shape:

- Temperature
  - Mass transfer and partitioning of analyte between mobile and stationary phase
  - Mobile phase viscosity
- Linear velocity, *u* of mobile phase
  - Higher *u* yields sharper peaks…
    - But retention may suffer if u is too fast.
- Mobile phase pH… should be 2 pH units away from pKA of compound – an ionized compound will exhibit less tailing and sharper peaks. If mobile phase is close to pKA of analyte, double peaks might occur.

Van Deemter curves of column plate height (H) vs. mobile phase flow rate (u)

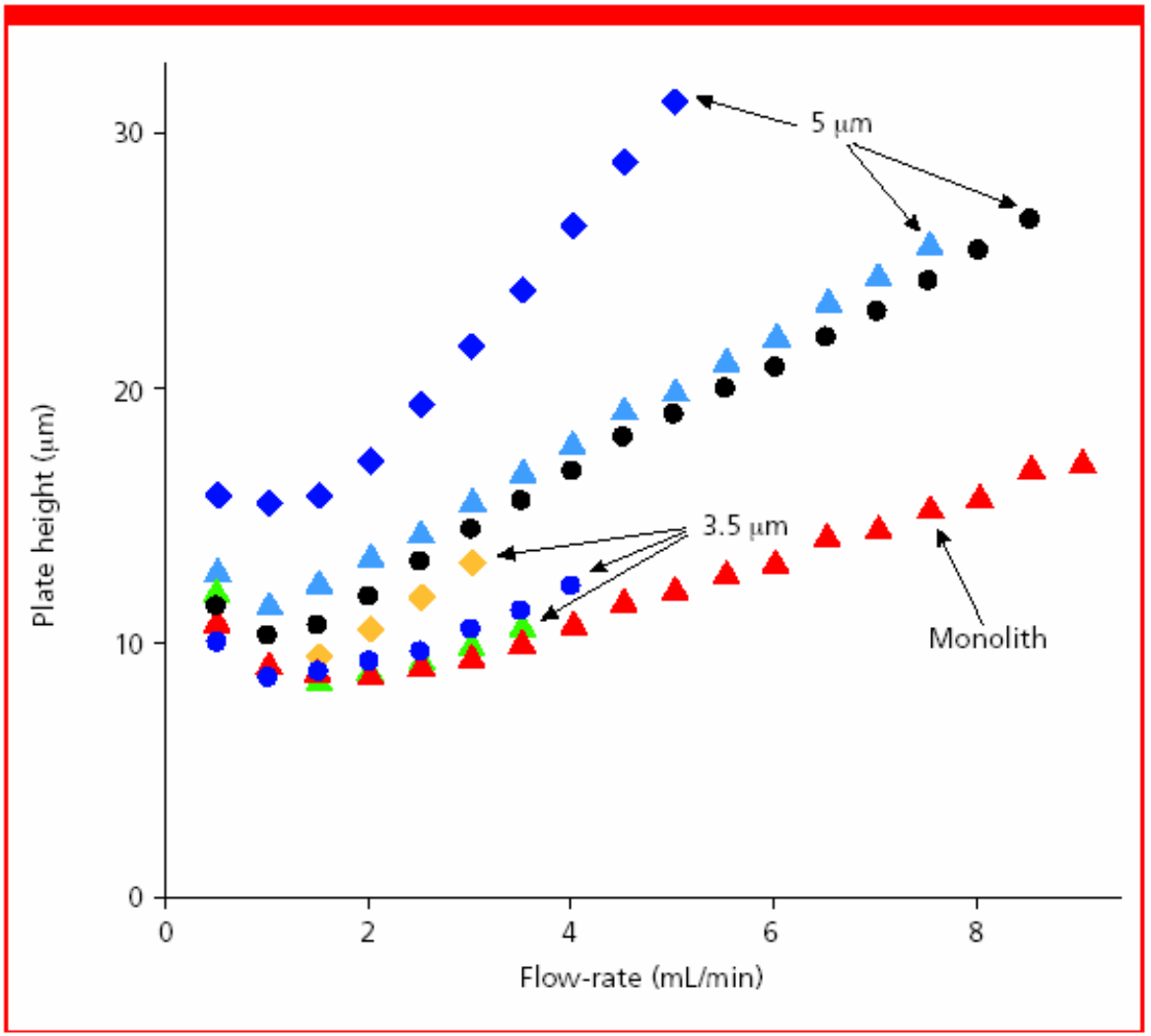


Figure 2: H *versus* u *curves for monolithic silica (Chromolith) and particulate HPLC columns.*

From: ***New Developments in the Application of Monolithic HPLC Columns,*** **LCGC Europe** - December 2001
Dieter Lubda, Karin Cabrera, Wolfgang Kraas, Christian Schaefer and Don Cunningham, Merck KGaA, Darmstadt, Germany, **Column Editor, Ronald E. Majors,** Agilent Technologies, Wilmington, Delaware, USA.

How can we apply the theory to achieve rapid separations?

A system must be carefully put together to achieve optimum separations. Following is a practical guide.

Heating system

- Mobile phase ideally will be heated pre injector and between injector and column.
- Various systems are out there. Column may be heated in a constant temp. water bath. (One Pre-heater that works is called "Caloratherm®" made by Selerity Technologies.)

Use high pressure components and fittings.

- Peek will pop out under pressure
- Stainless Steel should be installed carefully to avoid dead volume and to avoid breaking valves.

Mixer and System Internal Volume

- Keep system internal volume low! Use either a simple T connection or the lowest volume mixer you can find.
- Remove dampers from system. They add ½ to 1 mL of internal volume to system – deadly for fast LC!
- Eliminate excess tubing from system, but don't make it so extreme that you can't change columns, etc.!

Pay careful attention to ID of tubing *and* valves!

For 1.0 mm, 1.5 mm and 2.0 mm columns, use 0.005” ID Stainless Steel tubing for all plumbing from mixer to column. Valve ID should be 0.006” (0.005” ID valves are unavailable, but would be best. 0.004” ID valves are available, but too small – cause pressure increase in system.)

Use only pre-cut and electropolished SS tubing.

- Examine ends under low power microscope to confirm perfect cuts. – if not… discard.
- Ends must be cut perfectly square. – if not… discard.
- Ends must be flat with no ridges or imperfections. – if not… discard.

Column Backflushing

- Backflushing is not possible on all columns – check with manufacturer.
- Gradient backflush with acetonitrile, methanol, water and 10% acetic acid.

Heart cut injections

- This type of injection is done on the Waters Acuity™ system. It may be adapted to other injectors such as the Leap HTC Pal™ Autosampler.

Use a small injection volume.

- Large injections cause band broadening.
- The ideal injection volume is very small, and very concentrated. Sample should not be so concentrated it will crash into solution before it is adsorbed at the head of the column.

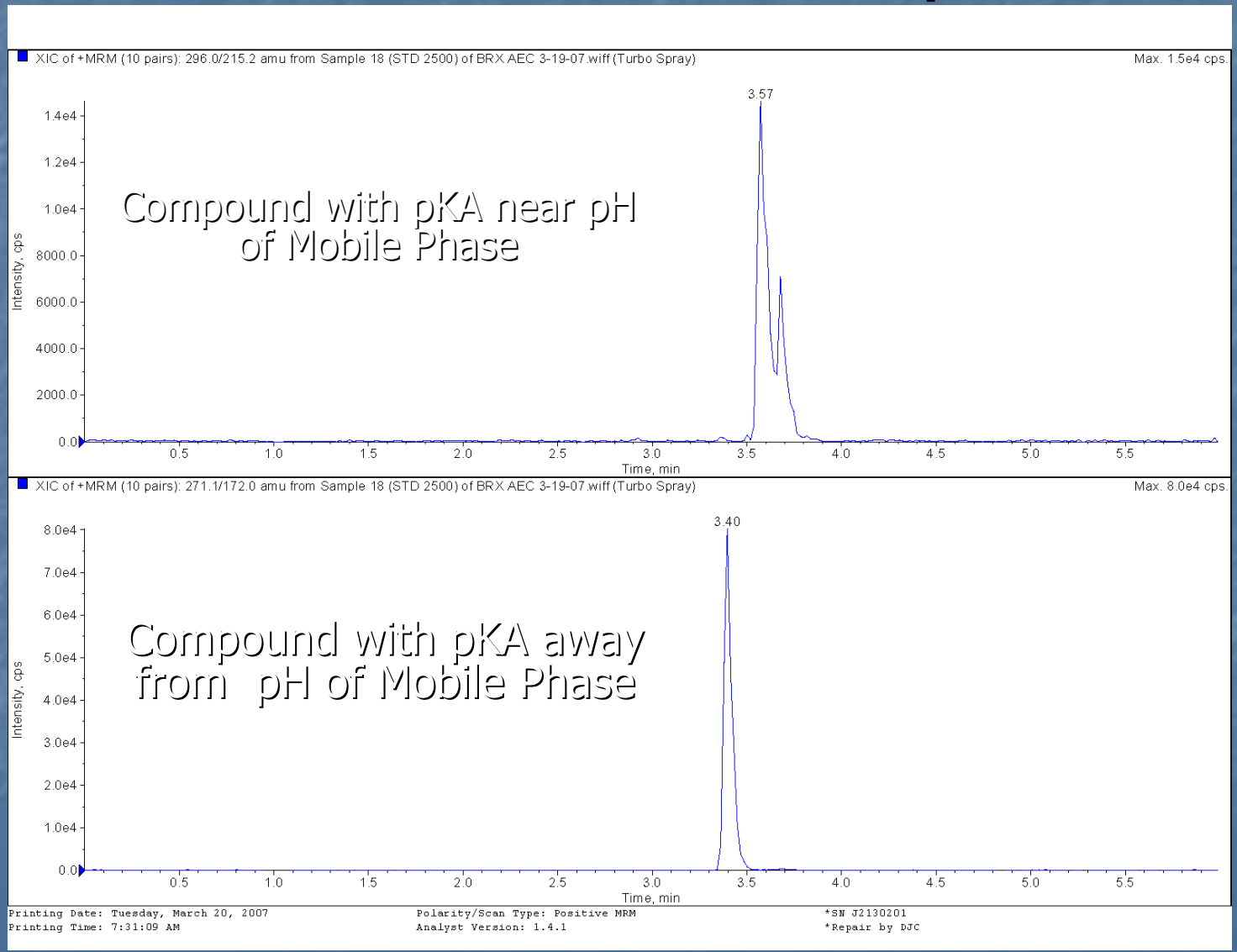
Injection Matrix

- Should ideally match mobile phase A
- For small volume injections, matrix becomes less important.
- Good peaks are possible even with a high organic injection matrix if injection volume is small.

Use pH as a tool for good separations

- HPLC techniques using Silica and phases such as C-18 or C-8 bonded to Silica, even 10 years ago had a much narrower usable pH range of 3 to 7.
- Contemporary stationary phases often have a usable pH range from 1 to 10. (This is an enormous benefit in achieving good separations.)
- Mobile phase pH should be 2 pH units away from the Analyte's pKA. (This avoids tailing, broad peaks and double peaks.)

Normal Peak and Split Peak, same run, 2 compounds



pH continued

- Use acid mobile phases for basic molecules (most drugs).
- Use basic, high pH mobile phases for acidic molecules (some drugs).
- If there is both an acidic and a basic site on the molecule, use an acidic mobile phase. The basic site will be ionized and the acidic site will stay neutral, as if it were not even there. Acidic mobile phases are easier on the column.

Typical Organic Mobile Phase (RPLC)

- Organic System:
  - 1st choice… pure Acetonitrile
  - 2nd choice… 50/50 Acetonitrile/Methanol
  - 3rd choice… pure Methanol
  - For difficult separations… try adding IPA or Ethanol (non-denatured)
  - Usually, no need to acidify or basify Organic Mobile Phase.

Mobile phase Aqueous Buffer Systems

- Trifluoroacetic acid pH 1.5-2.5
  Use 0.02% TFA (but avoid if possible – stays in system and causes ion suppression).
- Ammonium formate pH range 2.8-4.8, and 8.2-10.2
  Use 2 to 5 mM Ammonium Acetate with 0.3 % Formic Acid
- Ammonium acetate pH range 3.8-5.8, and 8.2-10.2
  Use 2 to 5 mM Ammonium Acetate with 1.0 % Acetic Acid
- Formic Acid, pH 1.89, Use 0.3% Formic Acid
- Acetic Acid, pH 2.38, Use 1.0% Acetic Acid

Mobile phase Aqueous Buffer Systems Continued

- Ammonium Acetate, use 2 to 5 mM
- Ammonium Acetate with Base, 8.2 – 10.2 pH, titrate w/ Ammonium Hydroxide, use 0.1% Ammonium Hydroxide in 10 mM Ammonium Acetate. pH is 8.75.
- In-between pH – Start with low pH while stirring and measuring realtime with pH meter – add base to solvent until desired pH is reached. If it's in the buffer range, pH will change slowly. Outside buffer zone, rapid jumps in pH are to be expected.

Water

- Don’t use bottled reagent water – it is often contaminated with bacteria.
- Build and maintain a good high purity water system:
- Feed water either distilled water or pre-purified before it enters MilliQ system.
- UV light bacterial growth suppression in holding tank.

HPLC Columns

- Not made equal.
- Efficiency varies greatly (by factor of about 5) between manufacturers.
- Given same efficiency, back-pressure also varies greatly.
- Thick column walls impede efficient heat transfer.
- The only way to judge a column is to test it and compare with others.

Summary

- Build a tight system – no dead volume and consistent in ID.
- Heart cut injection. Small injection.
- Heating system design is crucial.
- Use the best possible HPLC columns
- Use high quality reagents/buffers.
- Insure system has a small internal volume.
- Adjust pH, buffers and column to your analyte for good peak shape, good retention and good separation.

Thank you!

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