Use of Automation to Achieve High Performance SPE

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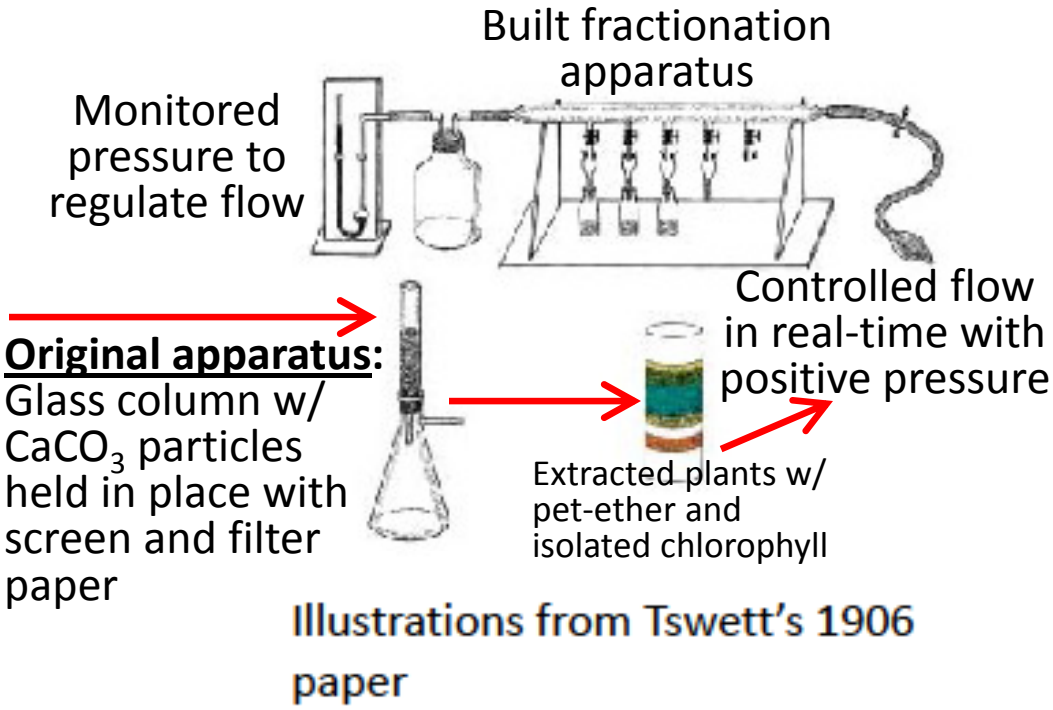
Key take away:
Solid phase extraction is liquid chromatography!
Operate it as such and get better results!

Chromatography: started with Tswett



Illustrations from Tswett's 1906 paper

While he didn't know why, Tswett knew importance of regulating flow when performing chromatography 110 years ago

Tswett's original apparatus is way many perform SPE today: 110 years ago, it was known that it needed to be done better!

Unfortunately, Tswett received little attention for this…

Chromatography received little attention until

- A.J.P. Martin and R.L.M. Synge described partitioning model explaining paper chromatography in 1941 (general mechanistic understanding of chromatography - Nobel)
- Erika Cremer and Fritz Prior built first GC in 1945
- Metal tubes filled with activated carbon used in large volume water sampling (emergence of SPE in 1950: "50 years of SPE", I. Liška, J. Chromatogr., A, 2000, 885, 3–16.)
- J.J. van Deemter , F.J. Zuiderweg, A. Klinkenberg, Chem. Eng. Sc., 1956, 5, 271–289 allowed understanding of chromatographic flow

Then, the basic knowledge was in place for the use of chromatography to grow exponentially

Evolution of Practical LC and SPE

- Commercialization of LC by J. Waters in the 1960s led to development of pumps, injection systems, & functionalized silica sorbent particles in the 1970s, similar to current use
- Parallel developments also led to SPE devices (syringe tube) in 1970s (96 well in 1990s, pipette tip in 2000s) still in use
- Development of HPLC closely followed teachings of van Deemter & Giddings (short diffusion distance & accurate flow)
- SPE development did not follow these teachings since it is not isocratic (required assumption to derive chromatography equations)
- Later, lessons of van Deemter & Giddings applied to gradient separations, inc. accurate measurement of plate count (Neue, UD, HPLC Columns: Theory, Technology, and Practice, Wiley, 1997, p77)
- Until now, single use SPE devices haven't followed these teachings, particularly importance of carefully controlled flow

Issues with common single use SPE devices

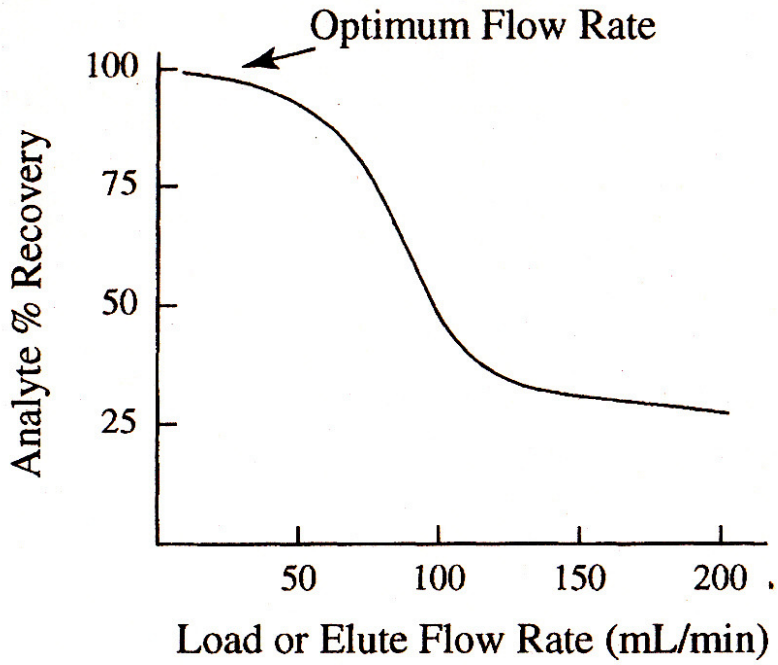
- Lack of flow control
  - With vacuum or pneumatic pressure, changing volume (weight) of liquid above sorbent changes flow rate during each SPE step
  - When performed in parallel for high throughput, effect is exacerbated since each SPE device (or well) has different resistance to flow & different variable flow rate
- Result of flow variability is considerable variability in absolute recovery (50-85% common) & results
  - Internal standards used to achieve meaningful results
  - Overall data evaluated based on worst case scenario (flow far from optimal, low absolute recovery)
- High absolute recovery against external standards in only solvent, the gold standard in demonstrating absence of matrix effects, isn't used with SPE

Assertion: SPE is LC

- Fundamentals of achieving high performance operation in LC: well packed sorbents to control variance in diffusion distance, precise flow control to match diffusion velocity/distance, & minimizing dispersion (dilution)
- History of use to improve column based LC performance is well documented & gains truly significant
- Despite >40 years performing SPE using LC sorbents, these principles have not been applied to SPE
- Reality: known principles that apply to gradient HPLC, apply to SPE equally

Typical single use SPE device performance

flow driven pneumatically or vacuum

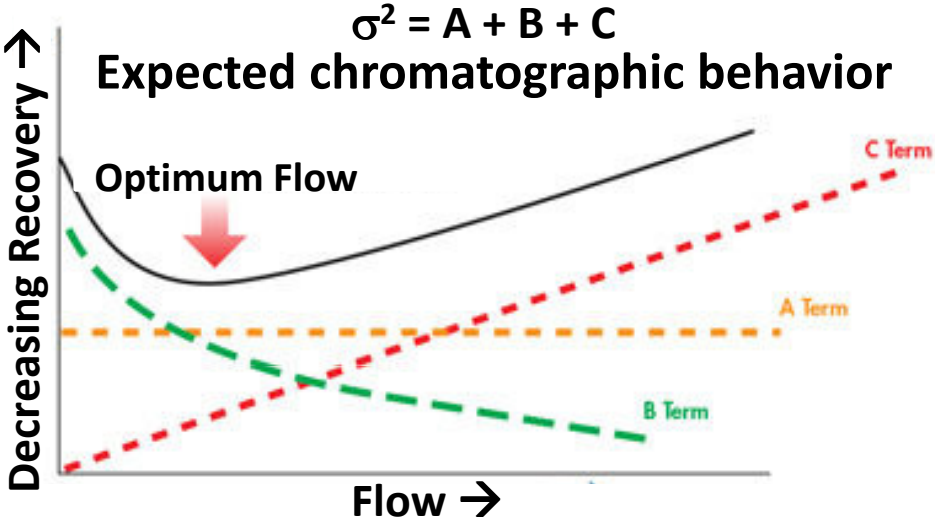


Jordan L, LCGC 1993, *11*, 634-8
for *huge* SPE cartridges

- Flow: slower is better but counter productive
- Lack of clearly defined optimum flow **demonstrates** lack of flow control or packed chromatographic sorbent, or both

<u>Commonly heard rationalization</u>: "SPE is digital chromatography." Thus, we cannot expect LC like performance despite the fact that we are using LC sorbents.

This presentation challenges that claim using packed sorbent & automation to achieve flow control.



Why do we use SPE?

- SPE is preferred tool for isolating target analytes from complex matrices due to availability of diverse range of chromatographic sorbents enabling targeted approaches based on specific chemistry of analytes & matrices
- Single use devices help prevent carryover
- SPE offers the ability to enrich or pre-concentrate analytes, particularly when drying & re-dissolving them afterward
- Enrichment is valuable allowing one to match analyte concentrations to approach used to measure them
- Given these unique capabilities, SPE often a first choice in analytical sample preparation

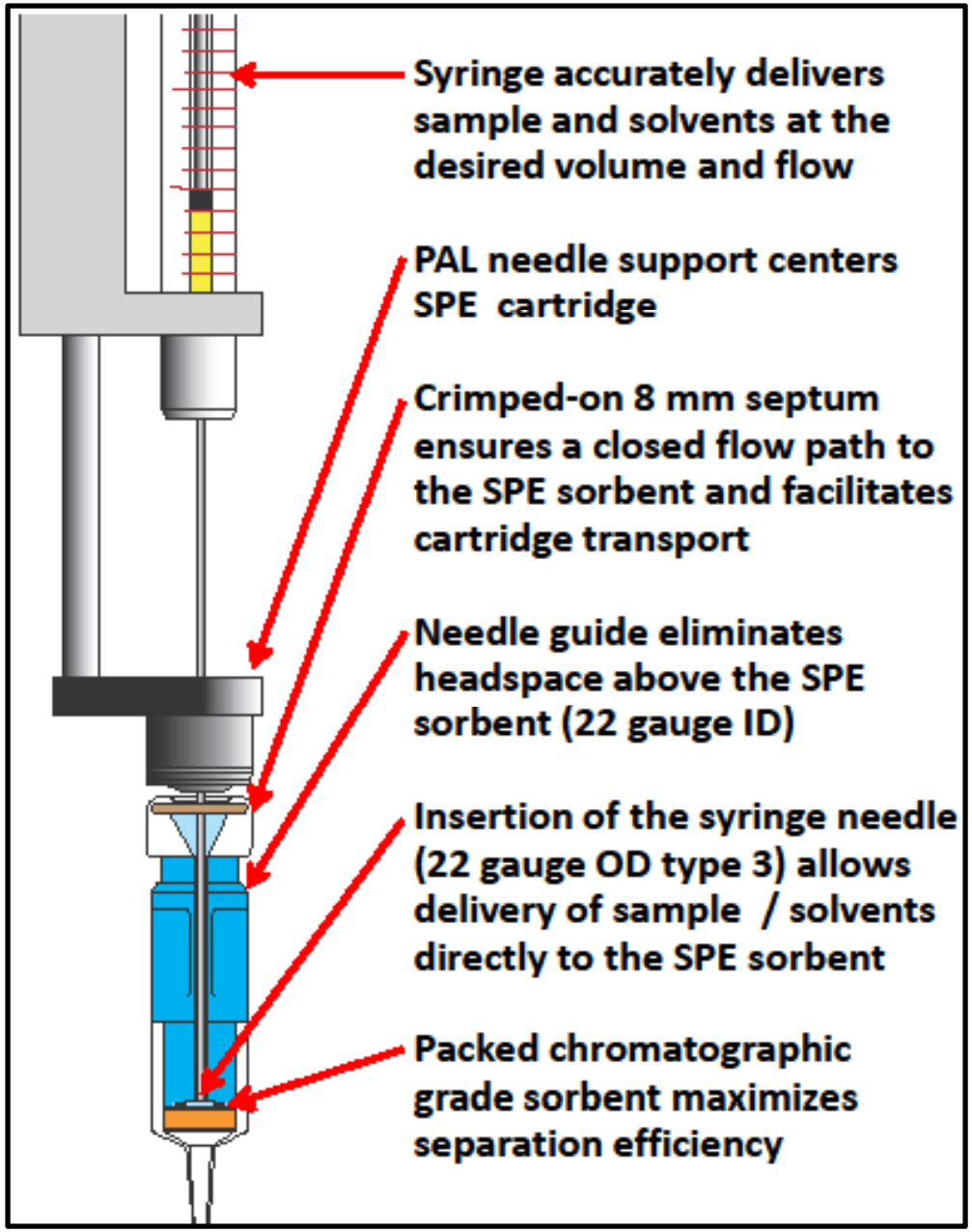
Testing a new micro-SPE device

- Initially, compelling reasons for interest in this new Smart SPE device from ITSP solutions were:
  - Automation using CTC/PAL autosampler performed at same cost as other single use SPE devices
  - SPE performed on-line in parallel with LC/MS/MS (or GC/MS/MS) analysis using CTC/PAL autosampler
  - Enrichment of analyte without need for drying eluant due to small elution volumes possible with this micro-SPE device
- Testing also showed interesting chromatographic performance not previously seen in other single use SPE devices

Heart of the ITSP form of SPE is patented *single use* cartridge containing customer-defined packed chromatographic media

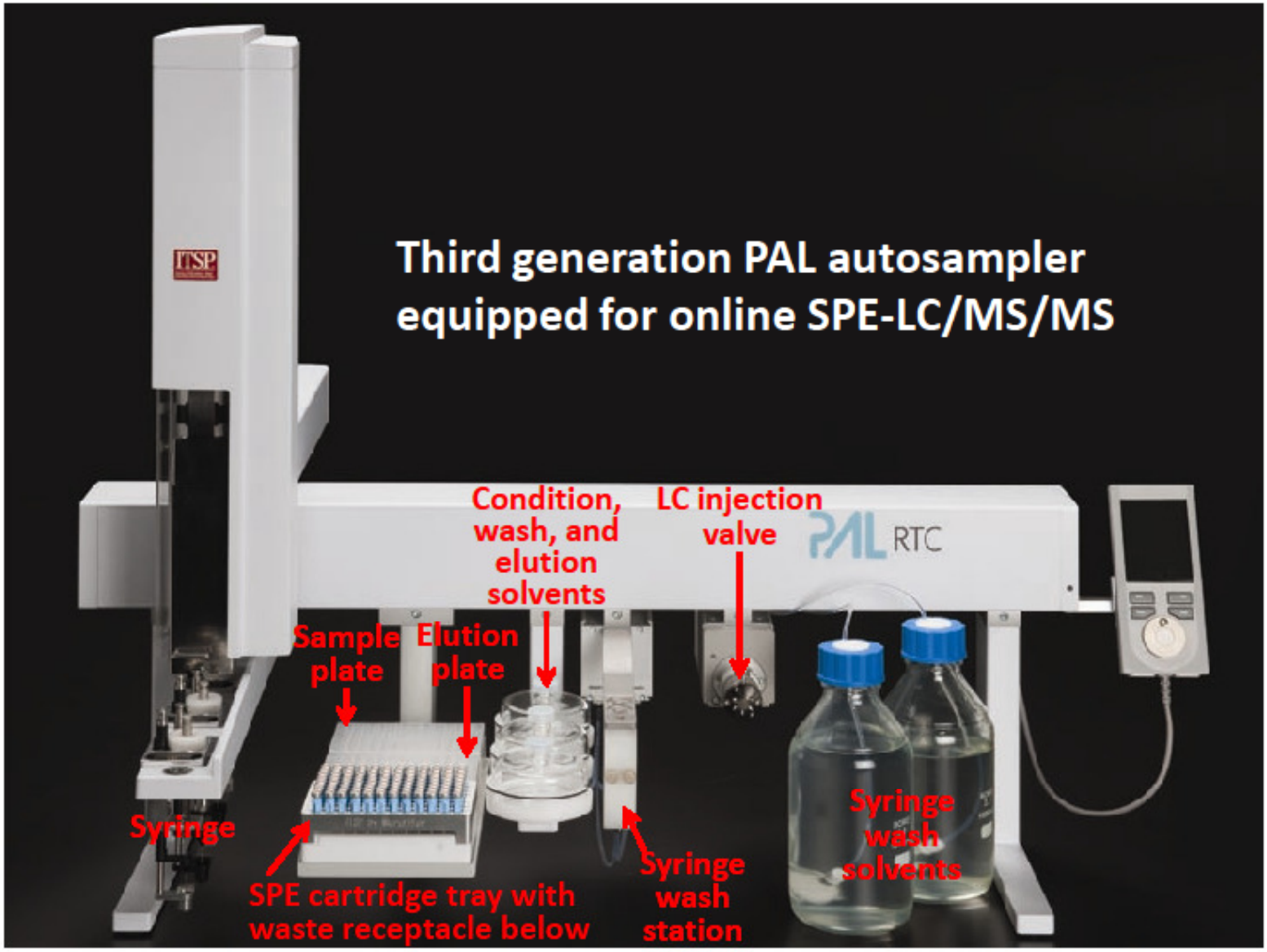
Crimped-on septum & needle guide (upper 80% of cartridge) enable automation by facilitating accurate cartridge transport on PAL syringe needle

The small (16 µl below sorbent) extra-column volume facilitates low volume elution (50-100 µl)



Automated and Higher Quality SPE Using an Autosampler: How it works

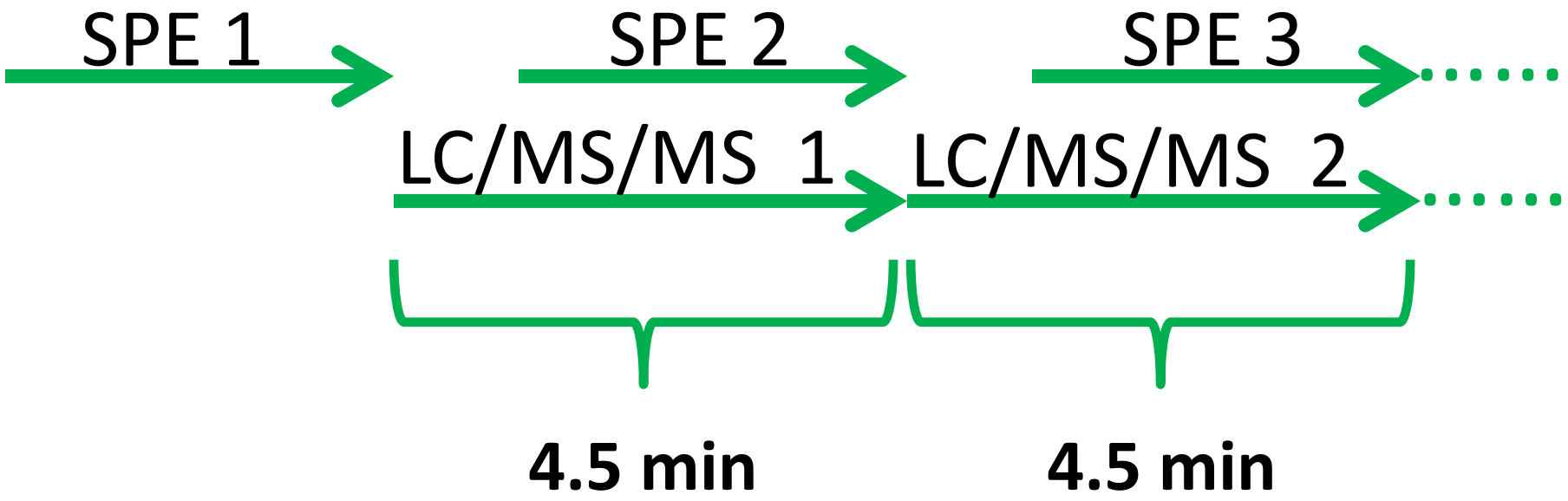
- Automated SPE begins using autosampler syringe for cartridge conditioning, sample loading & washing over waste receptacle
- Syringe then used to perform elution over clean vial or well, & used cartridge discarded in a different waste receptacle
- Automation complete after syringe mixes freshly eluted sample & then injects it into LC/MS/MS or GC/MS/MS used to measure the sample (see next Figure showing CTC/PAL autosampler).
- SPE performed in parallel after SPE of first sample



Hoses connect the SPE cartridge tray and syringe wash station to an ordinary lab solvent waste container. Used cartridges are typically discarded by the PAL into a box under the wash station and LC valve.

Workflow: minimizing cycle time

Parallel PAL operation in the inject ahead mode



Total cycle time (SPE + LC/MS/MS) = 4.5 min

Method: 71 drugs in urine, RP SPE – C18 – 50 μm particles

SPE time = 3.2 min, LC/MS/MS time = 4.5 min

Development of Automated SPE Methods Resulted in Detailed SPE Flow Studies

- UCT 50 μm C_{18} end capped silica sorbent
- Included in method development was thorough flow optimization study intended to measure cost/benefit in time/recovery

Confirmed results not previously expected:

- **The result was a 20 data point U-shaped curve showing that flow of 5 μl/s resulted in 100% absolute recovery**
- **Skeptical, the flow study was repeated, then again measuring 94 data points, then again measuring load and elute steps separately while holding the other at 5 μl/s**
- **All of these produced the same U-shaped curve and they all looked similar to a van Deemter curve**

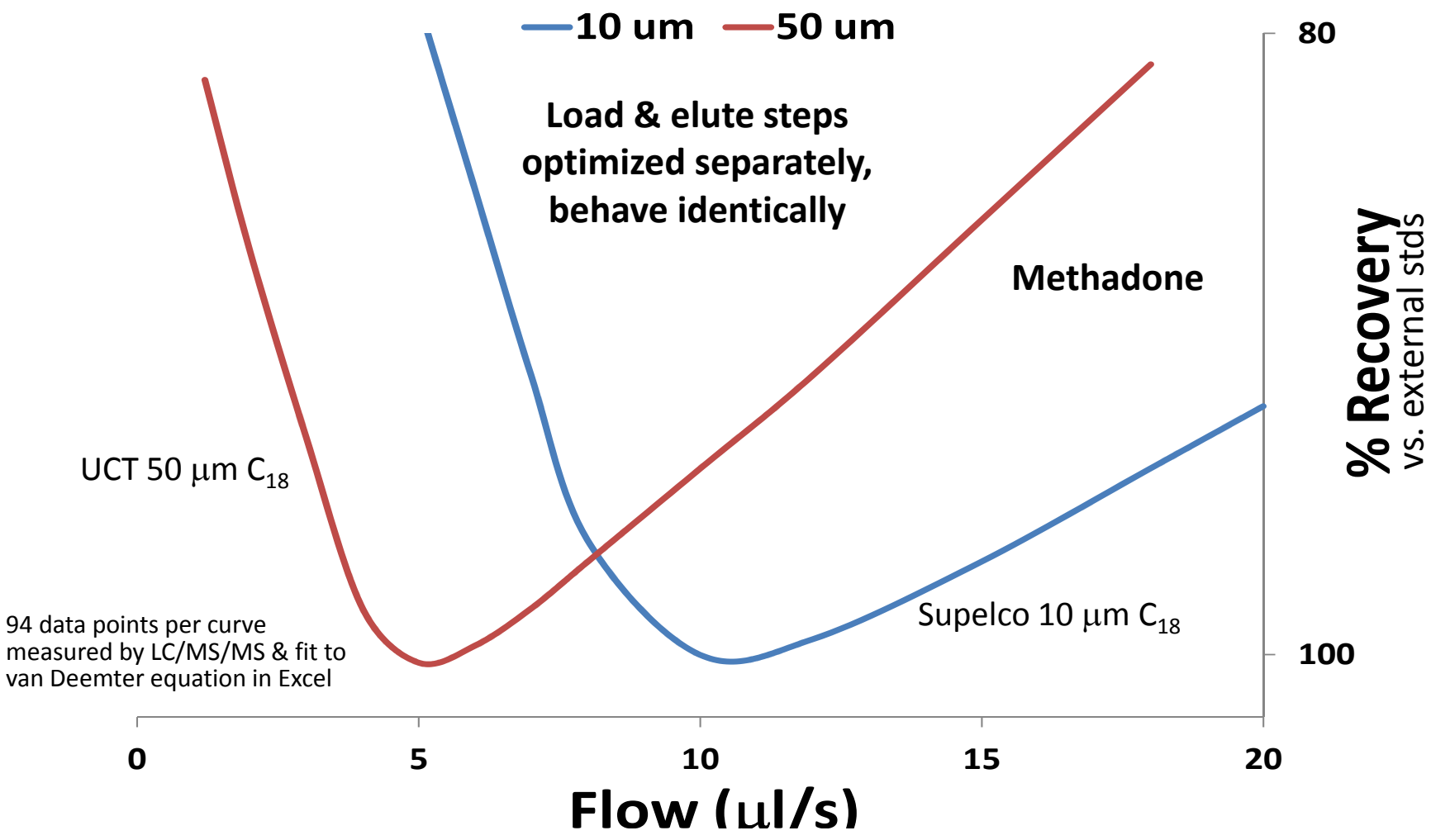
How to plot a van Deemter curve for SPE

- Conventional measures of SPE (recovery) differ from conventional measures of GC and LC (retention time & peak width [2σ])
- Yet the processes are same (diffusion, adsorption [or not], & desorption [or delayed]), the random walk model applies, & van Deemter equation is a variance (σ^2) equation [Giddings, JC, Unified Separation Science, Wiley 1991, p 92-101]
- Use of computerized chromatography data to evaluate separation performance [%RSD method for plate height calculation in Neue, UD, HPLC Columns: Theory, Technology, and Practice, Wiley, 1997, p12-13] offers simple view of relative nature of σ & how to address SPE data
- %Recovery measures deviation (σ) like LC peak width & thus, (100% - measured %Recovery)^2 is a measure of variance (σ^2)

Hence, plotting (100% - measured %Recovery)^2 vs. flow should yield typical van Deemter curve shape <u>if chromatographic processes govern dispersion of molecules in SPE</u>

Flow optimization for ITSP SPE:
just like LC column

van Deemter Curves for RP SPE
using 2 different particle sizes



Flow Optimization: Outcome and Impact

- **ITSP SPE cartridges behave like LC columns due to:**
  - Accurate flow control from PAL autosampler (syringe pump)
  - Sorbent is packed
  - Low extra-column volume
- **Benefits:**
  - >99% absolute recovery systematically achieved (within measurement precision of LC/MS/MS ±3%)
  - Same tools used to increase speed & performance for LC can be used for SPE (eg. ***smaller particles***)
  - Thus far, we haven't found an application that can't be done with 10 mg sorbent: *high sorbent mass SPE cartridges appear to be band-aid for overcoming low recoveries caused by insufficient flow control…*

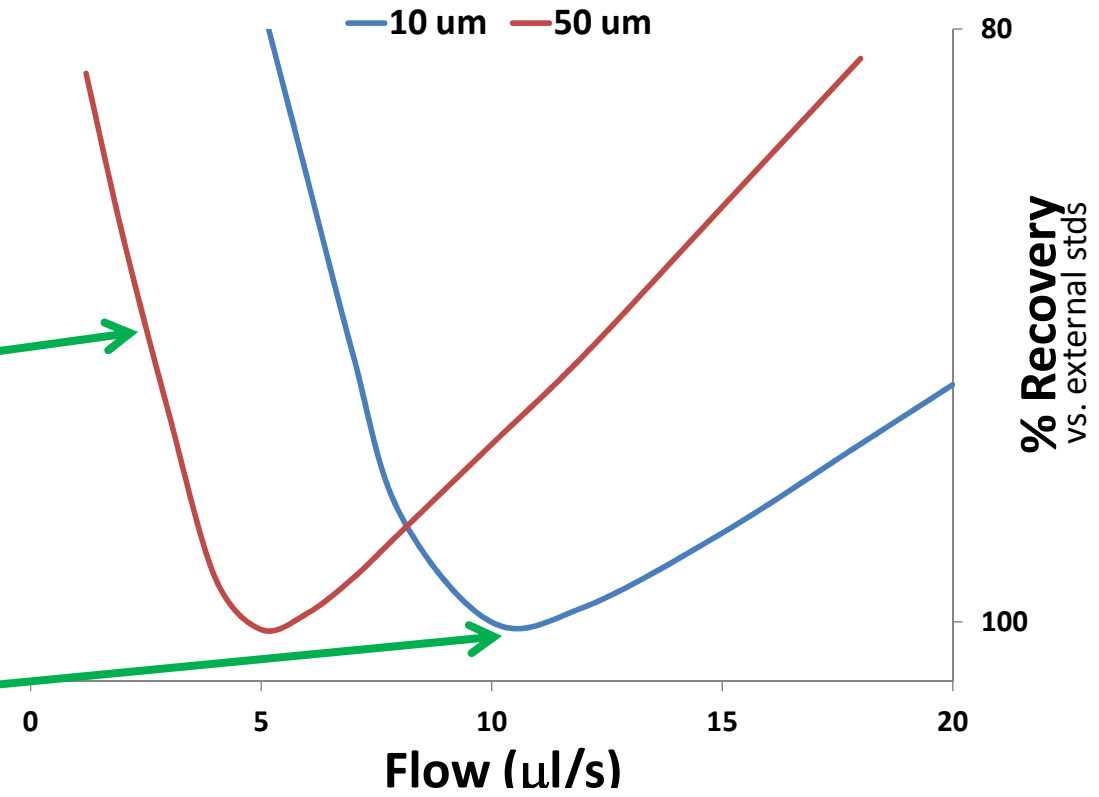
Use of chromatographic SPE knowledge

Same SPE & LC/MS/MS method except sample volume

- 71 drugs LC/MS/MS time = 4.5 min
- **SPE urine:** 200 µl sample loaded, 50 µm particles, SPE time = 3.2 minutes
- **SPE oral fluid:** 1000 µl sample loaded, 10 µm particles, SPE time = 4.5 minutes

van Deemter Curves for RP SPE

using 2 different particle sizes



SPE gets same benefits of particle size as LC (systematic control of speed)

SPE: Drugs in Urine and Oral Fluids

Same C_{18} RP method: different sample volumes loaded on SPE cartridge

Urine:

- Enrichment: 3x (200 μl load /75 μl elute MeOH)
- Cutoffs (all): ≤1 ng/ml (S/N=20+)
- 1 mg/day benzos, opioids, & metabolites easily measured (considered challenging)
- 192 samples/day/LCMSMS (50 μm - overnight only – typical small to medium lab workflow)
- Removes: salts (~2%), small organic acids/bases (~1%), sugars (oxidized and intact), amino acids, glucuronidase
- **Maintenance:** reagents/solvents & instrument PM / LC column change each 6 months without loss of performance

Oral fluid:

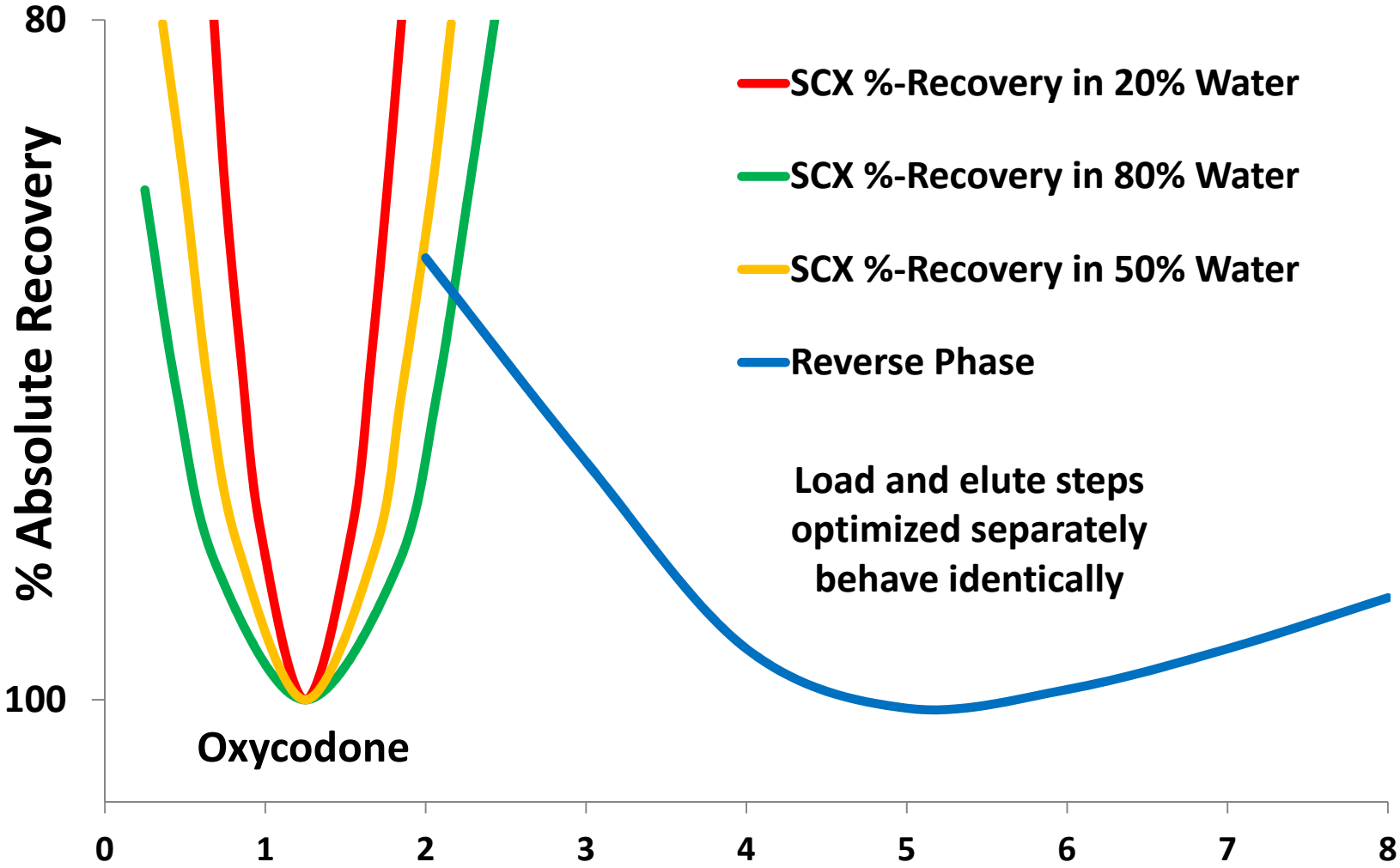
- Enrichment: 13x (1000 μl load /75 μl elute MeOH)
- Cutoffs (all): ≤ 0.2 ng/ml (S/N=20+)
- 1 mg/day benzos & metabolites easily measured (ordinarily considered not feasible)
- 192 samples/day/LCMSMS (10 μm particles - overnight only)
- Quantisal sampling/filtering (sample volume +/-10%)
- Removes: salts (~100 mM), mucopolysacharides, enzymes, glycoproteins
- **Maintenance:** reagents/solvents & instrument PM / LC column change each 6 months without loss of performance

Validated for 71 drugs – used for production >2 yrs

Further Study

- Rapid progress developing urine & oral fluid methods led to variety of method development efforts expanding the range analytes in more complex matrices (blood/tissues/food)
- To address the more complex matrices this led to use of more selective sorbents:
  - Cation exchange for drugs (mixed mode RP due to alkyl linker)
  - Anion exchange for lipids (mixed mode RP due to alkyl linker)
  - Chelation for phospholipids and phosphopeptides
- As might expected, this led to more SPE flow studies

Flow optimization for Cation Exchange SPE (50 μm particles)



n-propyl linked phenyl-SO_3^- on silica (UCT)

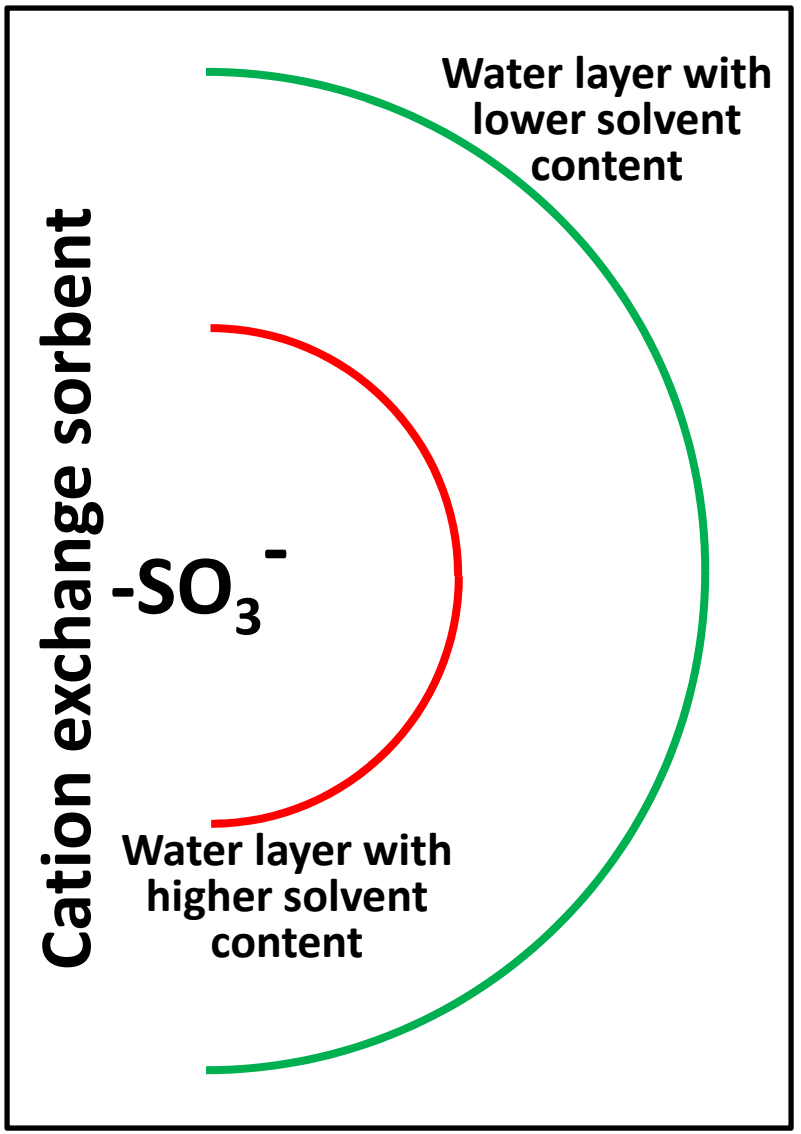
Molecules without cationic sites have optimal flow of 5 μl/s (i.e. RP)

Ionic SPE Flow Optimization: Outcome and Impact

- **Outcomes:**
  - Cation exchange SPE has optimum flow of 1.2 μl/s (1/4 that of reverse phase, 50 μm particles)
  - In addition, the relatively narrow acceptable flow range for high recovery widens with additional water content
  - Preliminary results suggest anion exchange and chelation SPE have the same van Deemter curves as cation exchange
- **Benefits:**
  - 100% recovery is systematically achieved (within the measurement precision of the LC/MS/MS ±3%) [rare with these chemistries]
  - Knowledge of the chemical preference for more water (less solvent) leads to more predictable and consistent outcomes
    - Use silica (not polymer) based particles to minimize solvent levels (n-propyl phenyl linker still gives sufficient mixed mode [RP] behavior)
    - Use strong miscible solvents (IPA/THF) to maximize water content

A possible explanation for water dependence on ionic adsorption / desorption

- Seems likely ionic sorbent sites have layer around them more concentrated with water (rather than solvent)
- Likely reason cation, anion, & chelation have the same optimum flow while having different binding energies (mass transfer regulated by transport through the water layer)
- Amount of solvent present regulates the thickness of water layer
- High performance SPE facilitates learning

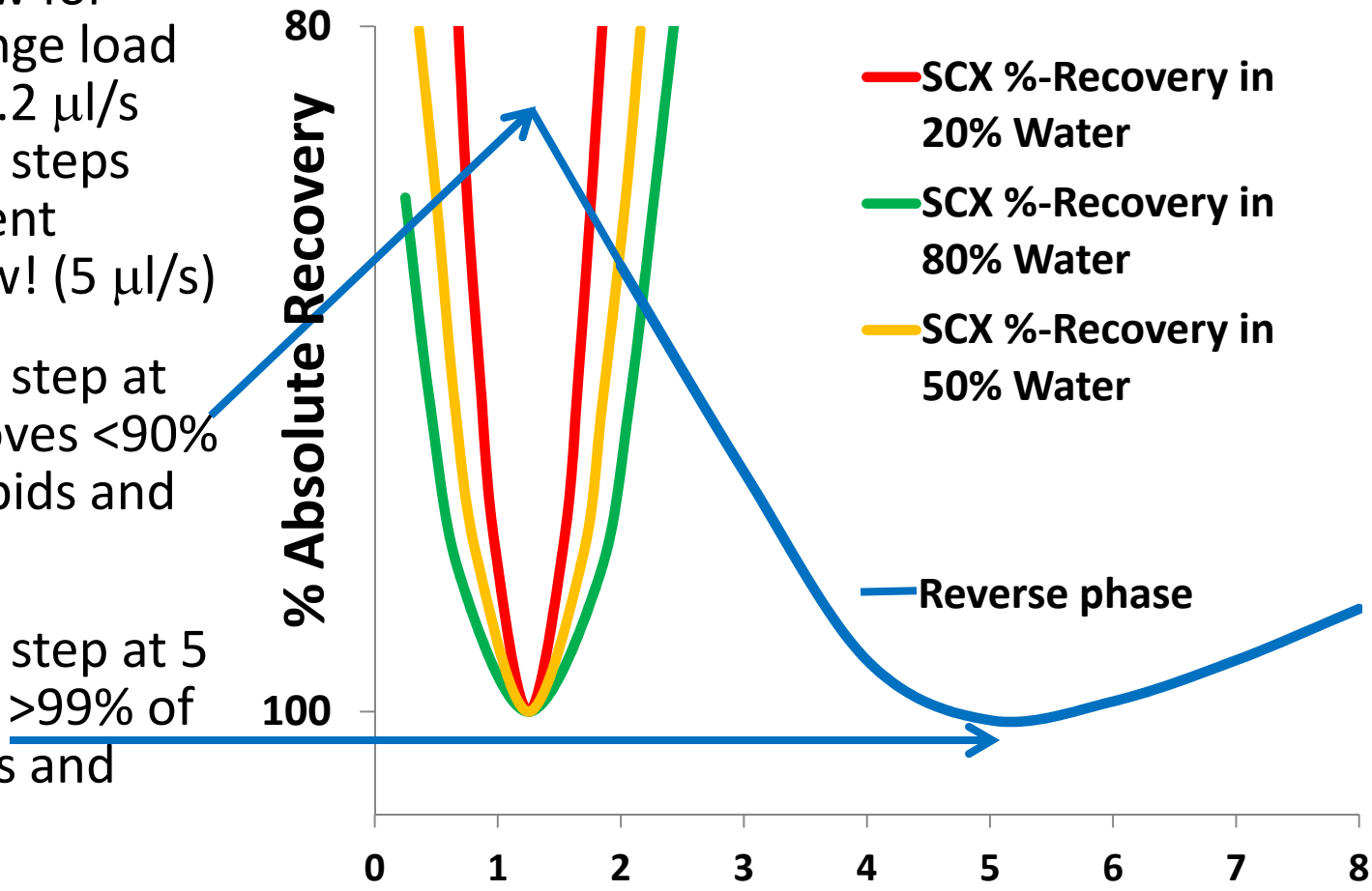


Use of chromatographic SPE knowledge

Cation exchange of basic drugs in blood (always mixed mode, 50 μm particles)

- Optimum flow for cation exchange load and elute = 1.2 μl/s
- Solvent wash steps have a different optimum flow! (5 μl/s)
- Solvent wash step at 1.2 μl/s removes <90% of phospholipids and fatty acids
- Solvent wash step at 5 μl/s removes >99% of phospholipids and fatty acids

Flow chosen depends on chemistry performed!



With 1.2 μl/s solvent wash, phospholipids are observable by –ion full scan LC/MS & not observable with 5 μl/s solvent wash

SPE: Basic Drugs in Blood

n-propyl linked phenyl-SO_3^- on silica - 50 μm

- Sample: 100 μl, 2/1 IPA crash, load 150 μl, 100 μl elute H_2O/IPA/NH_4OH)
- Washes: 0.02M pH 6 phosphate buffer, IPA/MeOH, H_2O
- Cutoffs (all): ≤1 ng/ml (S/N =20+), 100x better LogP ≥4
- 1 mg/day benzos, opioids, and metabolites readily measured
- 96 samples/day/LCMSMS (50 μm - overnight only – typical small to medium lab workflow, more possible)
- Removes: salts, organic acids, sugars (oxidized and intact), amino acids, **LIPIDS**
- >99% absolute recoveries
- 2D-LC (trap & elute) gives same performance for blood spots
- **Maintenance:** reagents/solvents , pre-column filter frit monthly (not needed with column switching), & instrument PM / LC column change each 6 months without loss of performance **(forensics)**

Validated for 63 drugs (inc cationic illicits)

Summary and Conclusions

- **With flow control and packed sorbent, SPE is gradient LC**
  - All existing knowledge about gradient LC can be applied to SPE
    - Smaller particles for RP SPE significantly increases speed without impact on performance
  - Applying this knowledge produces significantly better results than alternatives
    - 99+% recovery / matrix removal systematically achieved
- **Adsorption and desorption is a reversible equilibrium**
  - Separate measurement of load and elute flow behavior produces the same van Deemter curves (just like isocratic LC)
- **SPE using single use devices with accurate flow control can achieve high quality even in high throughput applications (fast automation)**

Given the same price point, it's hard to see rationale for continued use of single use SPE devices that utilize loose sorbent and/or vacuum / pneumatic driven flow