Use of Automation to Achieve High Performance SPE

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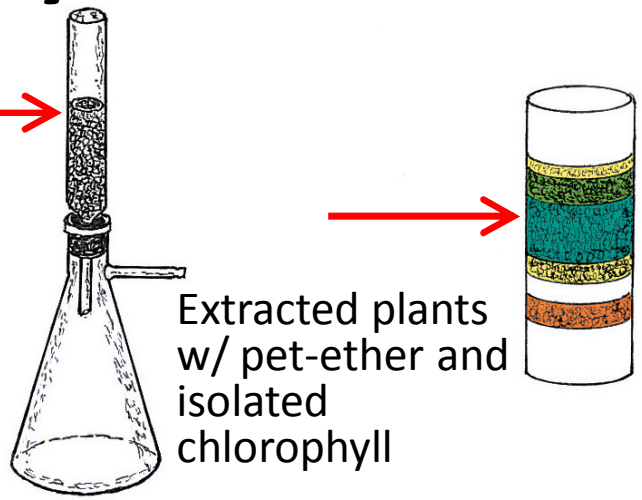
Key take away:
Solid phase extraction is liquid chromatography!
Operate it as such and get better results!

Chromatography: started with Tswett

Original apparatus:
Glass column w/ $CaCO_3$ particles held in place with screen and filter paper



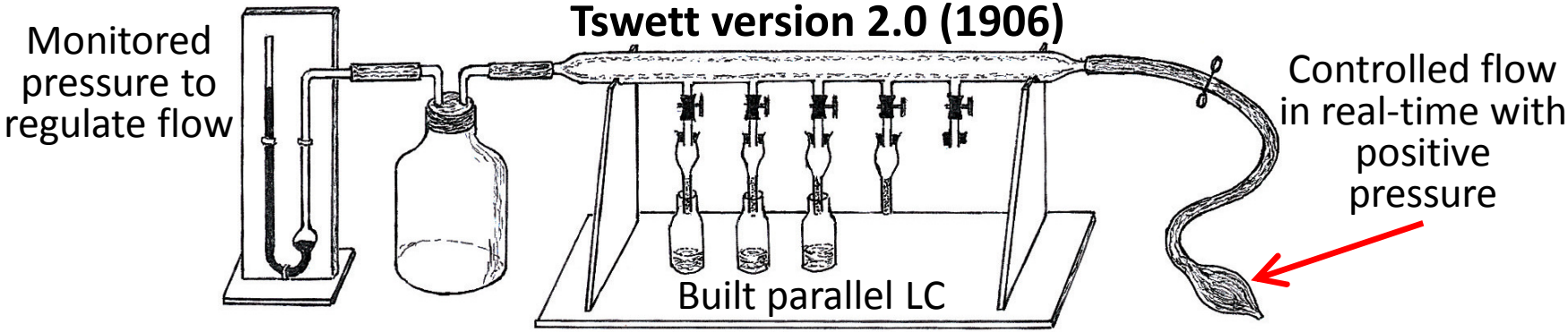
Extracted plants w/ pet-ether and isolated chlorophyll

Illustrations are photographs of Tswett's hand drawings

Tswett's original apparatus is the way many perform SPE today: >100 years ago, it was known that it needed to be done better!

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

Tswett version 2.0 (1906)



Unfortunately, Tswett received little attention for this…

MS Tsvet: Physical chemical studies on chlorophyll adsorptions, Berichte der Deutschen botanischen Gesellschaft 24, 316–323 (1906)

LS Ettre: M.S. Tswett and the Invention of Chromatography, LCGC 21(5):458-467 (2003)

Chromatography received little attention until…

- 1941: partitioning model explains chromatographic process (mechanistic understanding chromatography - Nobel)*
- 1945: Erika Cremer and Fritz Prior built first GC**
- 1950: Metal tubes filled with activated carbon used in large volume water sampling (emergence of SPE)***
- 1956: Quantitative understanding of chromatographic flow****

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

*AJP Martin, RLM Synge. Biochem. J. 1941, 35, 1358–1368.
*LS Ettre , "The birth of partition chromatograph" LCGC, 2001, 19, 506–512.
**F Prior, PhD Thesis, Univ Innsbruck, 1947.
***"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 .

Evolution of Practical LC and SPE

- Commercialization of LC efforts by J. Waters in the 1960s
    - Led to pumps, injection systems, & silica sorbent particles in the 1970s
- Silica sorbent development led to SPE (syringe tube) in 1970s
- Development of *HPLC closely followed teachings of van Deemter* & Giddings*** (1970s – 2000s) [accurate flow & small particles]
- *SPE development did not follow these teachings since it is not isocratic* (Required assumption to derive chromatography equations)
- Later, van Deemter & Giddings teachings were applied to *gradient* LC separations and shown to apply***

Until now, single use SPE devices haven't followed these teachings, particularly the importance of carefully controlled flow

*JJ van Deemter , FJ Zuiderweg, A Klinkenberg, Chem. Eng. Sc., 1956, 5, 271–289
**JC Giddings, Dynamics of Chromatography: Principles and Theory, CRC Press, 1965
*** UD Neue, HPLC Columns: Theory, Technology, and Practice, Wiley, 1997, p77

Issues with common single use SPE devices

- Lack of flow control
  - With vacuum, gravity, or pneumatic pressure, changing weight of liquid above sorbent changes flow
  - Changing solvent viscosity changes flow
  - In parallel, effect is exacerbated since each device has different flow resistance & variable flow rate
- Result of flow variability is variability in absolute recovery (50-85% is common) & thus in the results
  - ISs 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 solvent is highly desirable, but isn't achieved with SPE

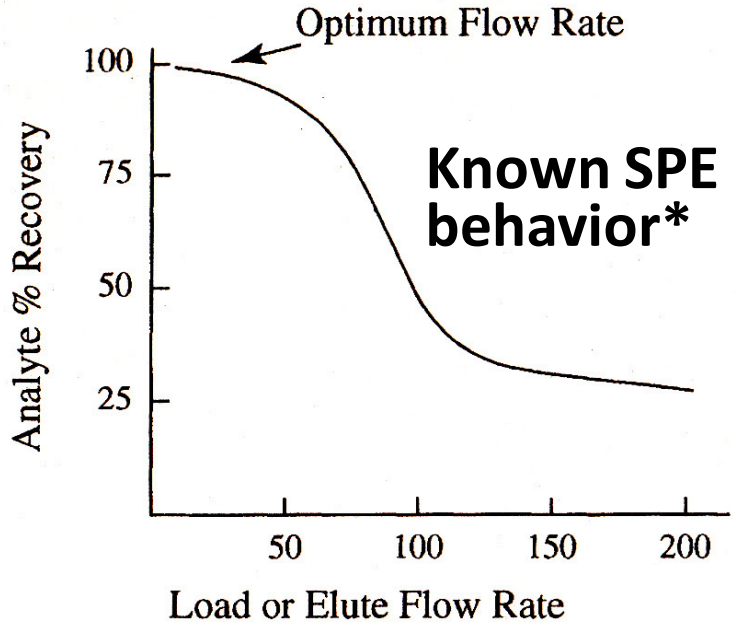
3 injection experiment proves no matrix effects:
gold standard in demonstrating absence of matrix effects

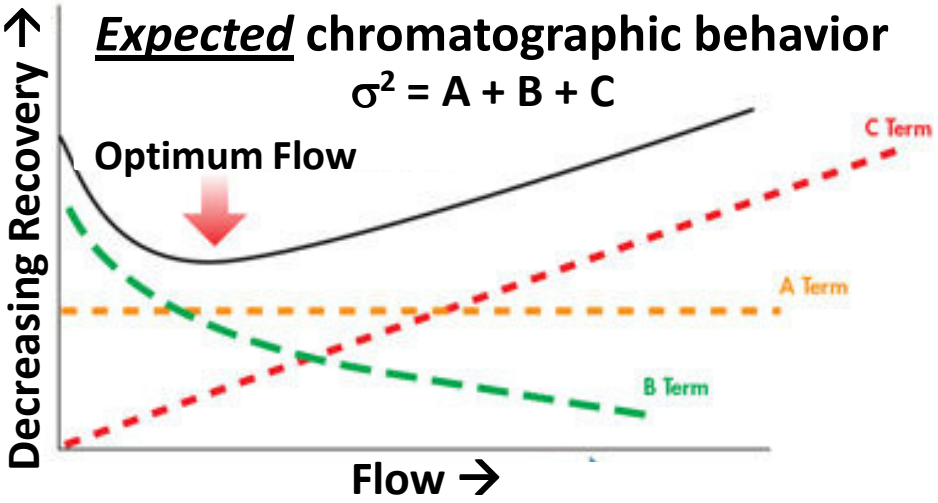
Assertion: SPE is LC!

- Fundamentals of 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 improving LC performance well documented & gains truly significant
- Until now, these principles have not been applied to SPE
- Commonly heard rationalization:
  - "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

Reality: all known principles that apply to gradient HPLC, apply to SPE equally

Typical single use SPE device performance





- Flow: slower is better but counter productive

Lack of clearly defined optimum flow *demonstrates* lack of flow control or packed chromatographic sorbent, or both

*Jordan L, LCGC 1993, *11*, 634-8

Why do we use SPE?

- SPE is a preferred tool for isolating target analytes from complex matrices due to:
  - Availability of diverse range of chromatographic sorbents
  - Enables targeted approaches based on specific chemistry of analytes & matrices ***(important)***
- SPE offers the ability to enrich or pre-concentrate analytes (particularly when drying & re-dissolving)
  - Enrichment is valuable allowing one to match analyte concentrations to approach used to measure them
- Single use devices help prevent carryover

Given these unique capabilities, SPE often first choice in analytical sample preparation

Compelling reasons for interest in SmartSPE

- Automation using CTC/PAL (Gerstel MPS) autosampler
- SPE performed on-line in parallel workflow with LC/MS/MS (or GC/MS/MS)
- JIT sample prep delivers “freshest” possible sample with no time cost for sample prep*
- No need for drying eluant due to small elution volumes (up to 200x enrichment)*
- Testing also showed interesting chromatographic performance not previously seen in other single use SPE devices*

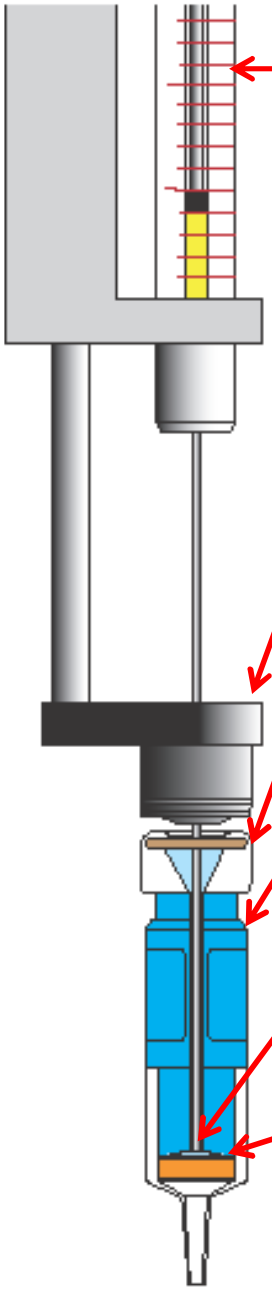
***Hayward M, et. al. Am. Lab., 2016, 48(7): 14-17**

Heart of SmartSPE form:

Single use cartridge containing customer-defined (no limits) packed chromatographic media

Crimped-on septum & needle guide enable accurate cartridge transport (automation)

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



Syringe accurately delivers sample and solvents at the desired volume and flow

PAL needle support centers SPE cartridge

Crimped-on 8 mm septum ensures a closed flow path to the SPE sorbent and facilitates cartridge transport

Needle guide eliminates headspace above the SPE sorbent (22 gauge ID)

Insertion of the syringe needle (22 gauge OD type 3) allows delivery of sample / solvents directly to the SPE sorbent

Packed chromatographic grade sorbent maximizes separation efficiency

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Automated SmartSPE Using an Autosampler: How it works

- Autosampler syringe conditions, loads sample & washes cartridge over waste receptacle
- Autosampler syringe transports cartridge to perform elution over clean vial or well, then discards cartridge
- Syringe mixes freshly eluted sample & then injects it into LC/MS/MS or GC/MS/MS
- SPE performed in parallel after SPE of first sample

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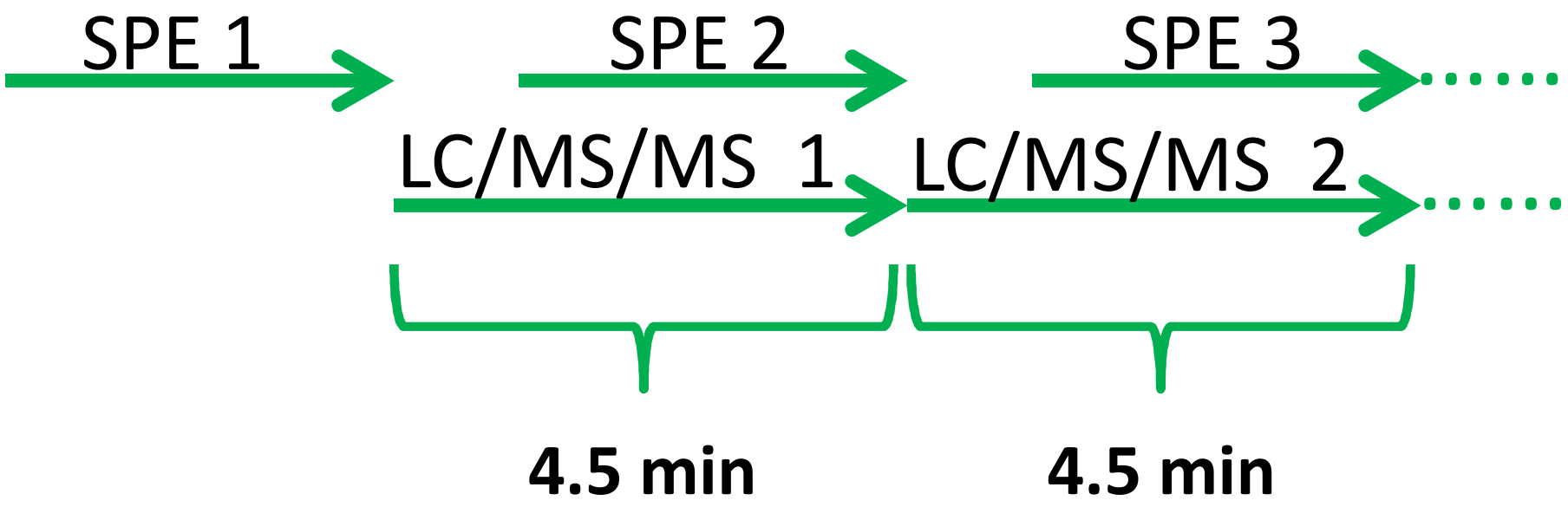
Workflow: minimizing cycle time

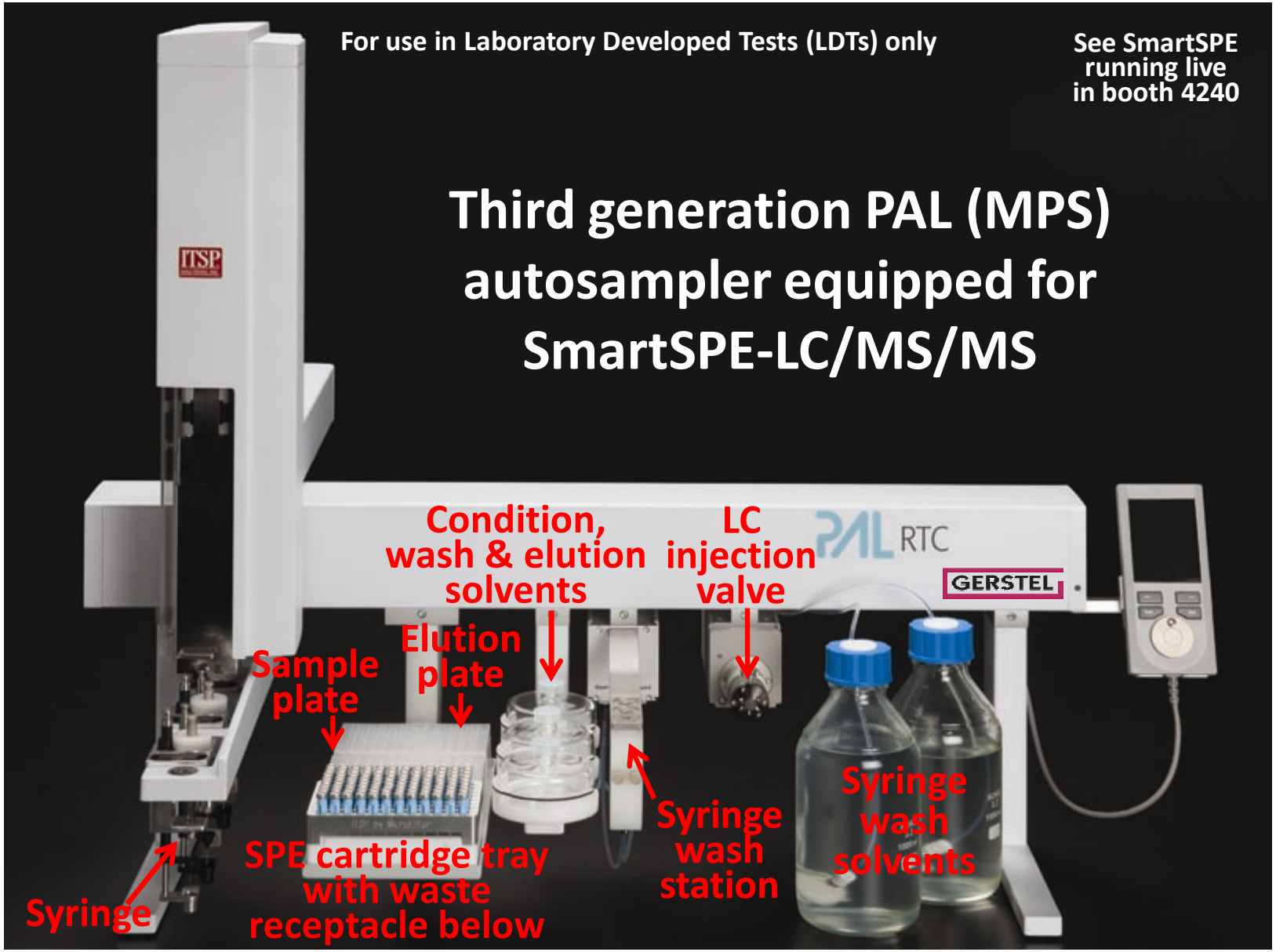
Parallel PAL (MPS) operation in the inject ahead mode



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



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 (MPS) into a box under the wash station and LC valve.

Want more methods? Add sample tray holders and solvents! → 4 SmartSPE methods without reconfiguration: No problem!

Development of Automated SmartSPE 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* & van Deemter equation is a variance (σ^2) equation
- Use of computerized chromatography data to evaluate separation performance 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)

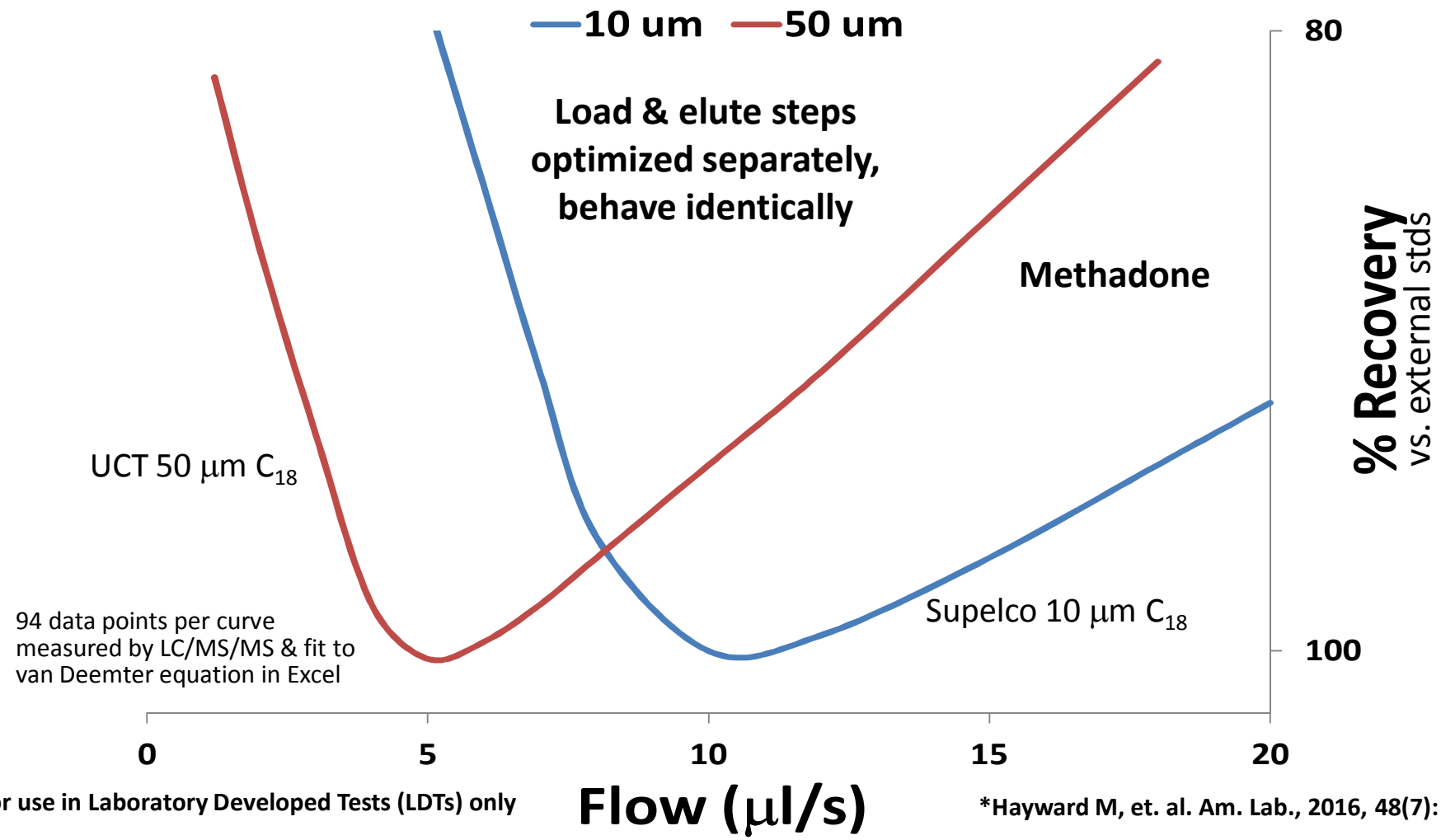
Hence, plotting (100% - measured %Recovery)^2 vs. flow should yield typical van Deemter curve shape if chromatographic processes govern dispersion of molecules in SPE

*Giddings, JC, Unified Separation Science, Wiley 1991, p 92-101 (diffusion, adsorption [or not], & desorption [or delayed]), the random walk model applies)

**Neue, UD, HPLC Columns: Theory, Technology, and Practice, Wiley, 1997, p12-13 (%RSD method for plate height calculation)

Flow optimization for SmartSPE:
just like LC column

van Deemter Curves for RP SPE
using 2 different particle sizes



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Flow Optimization: Outcome and Impact

- **SmartSPE cartridges behave like LC columns due to:**
  - Accurate flow control from PAL (MPS) autosampler (syringe pump)
  - Sorbent is packed
  - Low extra-column volume
- **Benefits:**
  - >99% absolute recovery systematically achieved (LC/MS/MS ±3%)
  - Smaller particles can be used to increase speed for SPE (just like LC)
  - Thus far, we haven't found an application that can't be done with 10 mg sorbent (MS detection)

High sorbent mass SPE cartridges appear to be band-aid for overcoming low recoveries caused by insufficient flow control…

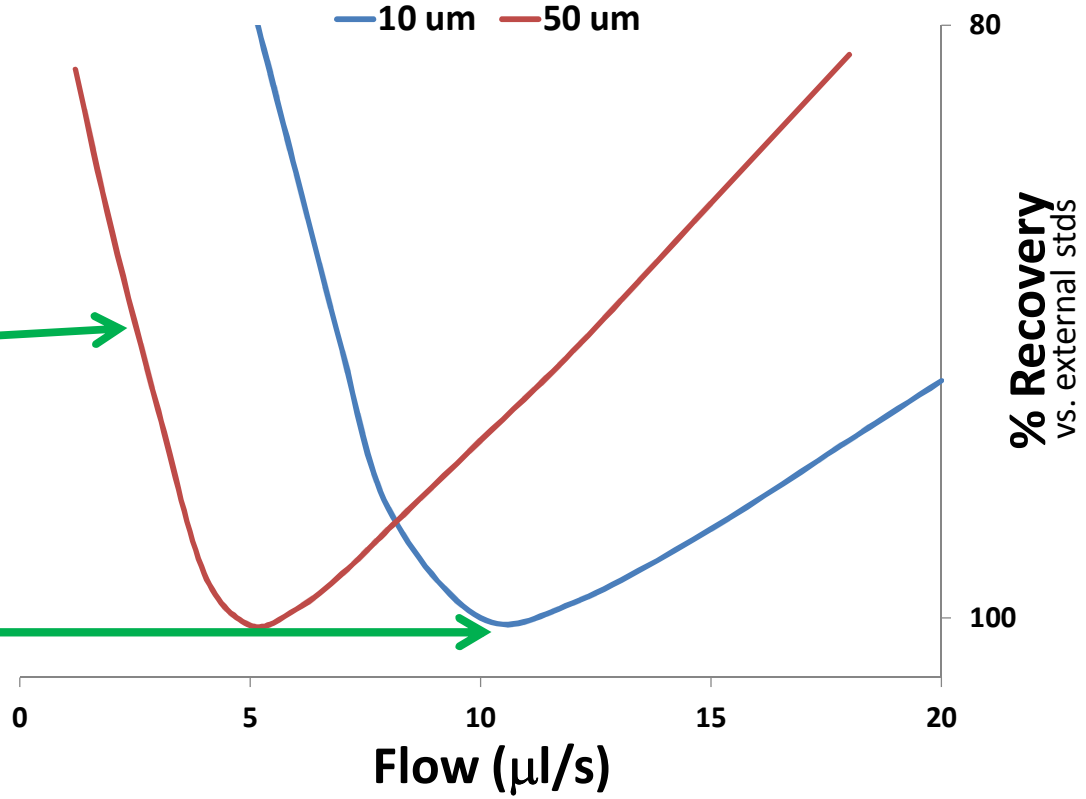
Use of chromatographic SPE knowledge

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
- Sample load time for oral fluid reduced from 2.6 min to 1.3 min

van Deemter Curves for RP SPE

using 2 different particle sizes



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

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SmartSPE: Drugs in Urine and Oral Fluids

Same C_{18} RP method: different sample volumes loaded on SmartSPE 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)
- **Matrix removed:** 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%)
- **Matrix removed:** 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 >3 yrs with typical mid-grade and econo QQQs

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App note at www.ITSPsolutions.com/application-notes

SmartSPE: Drugs in Urine and Oral Fluids

Impact on clinical labs (goal: maximize productivity)

- All human activities performed during daytime business hours & most measurements performed overnight
  - Overnight sample capacity is two 96 well plates per LC/MS/MS
  - Annual average overnight utilization rates of 90% can be achieved when samples per day ≥200
- With very good overall execution and sufficient scale, favorable economics can be achieved with overnight operation
  - Validated for 71 drugs: used for production >3 yrs with typical mid-grade and economy QQQs
  - 1000+ samples/day, 6+ LC/MS/MSs
  - ≤$30 total cost per sample versus $80 reimbursement
  - Robust: standard 6 mo PMs result in very high instrument “up times”
  - Repeats: physician request only, no outcome change
- Quality: QC & proficiency samples are 100% ID & observed within ±3% of actual concentration
- All above applicable to forensics (urine screen prior to blood work or oral fluid for DUID)

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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 SmartSPE (50 μm particles)

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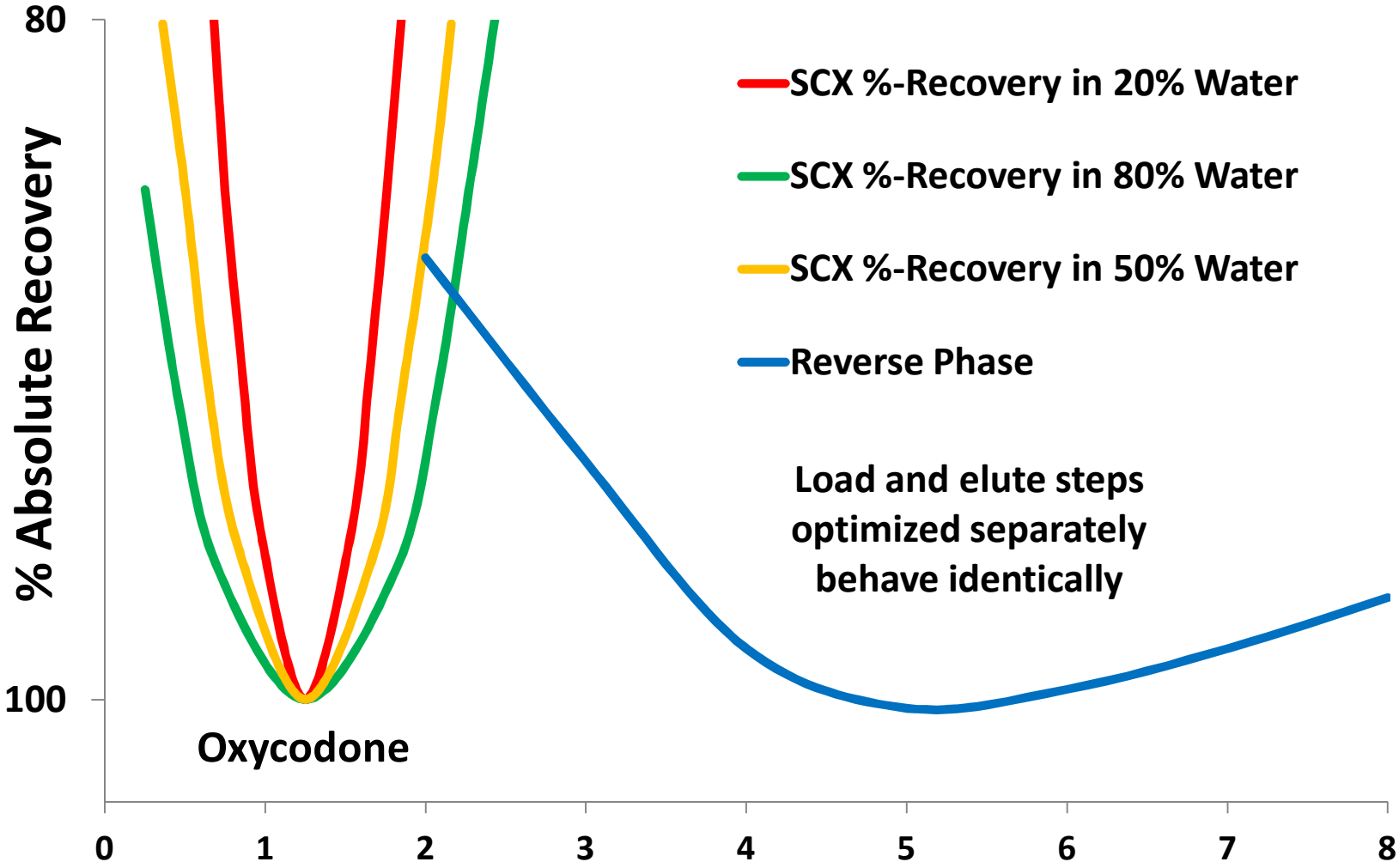

n-propyl linked phenyl-SO_3^- on silica (UCT)

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

Ionic SmartSPE Flow Optimization: Outcome and Impact

- **Results:**
  - Cation exchange SPE has optimum flow of 1.2 μl/s (1/4 that of reverse phase, both on same 50 μm silica particles)
  - Narrow acceptable flow range for high recovery widens with additional water content
  - Ionic strength based elution starts at 0.05M & is complete at 2M (approx)
  - Preliminary results suggest anion exchange and chelation SPE have the same van Deemter curves as cation exchange
- **Benefits:**
  - >99% recovery is systematically achieved (LC/MS/MS ±3%)
  - Knowing chemical preference for more water leads to more predictable and consistent outcomes
    - Minimize sorbent RP behavior and/or use strong miscible solvents: >90% solvent for load/elute lowers both ionic strength and recovery!

Use of chromatographic SPE knowledge

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

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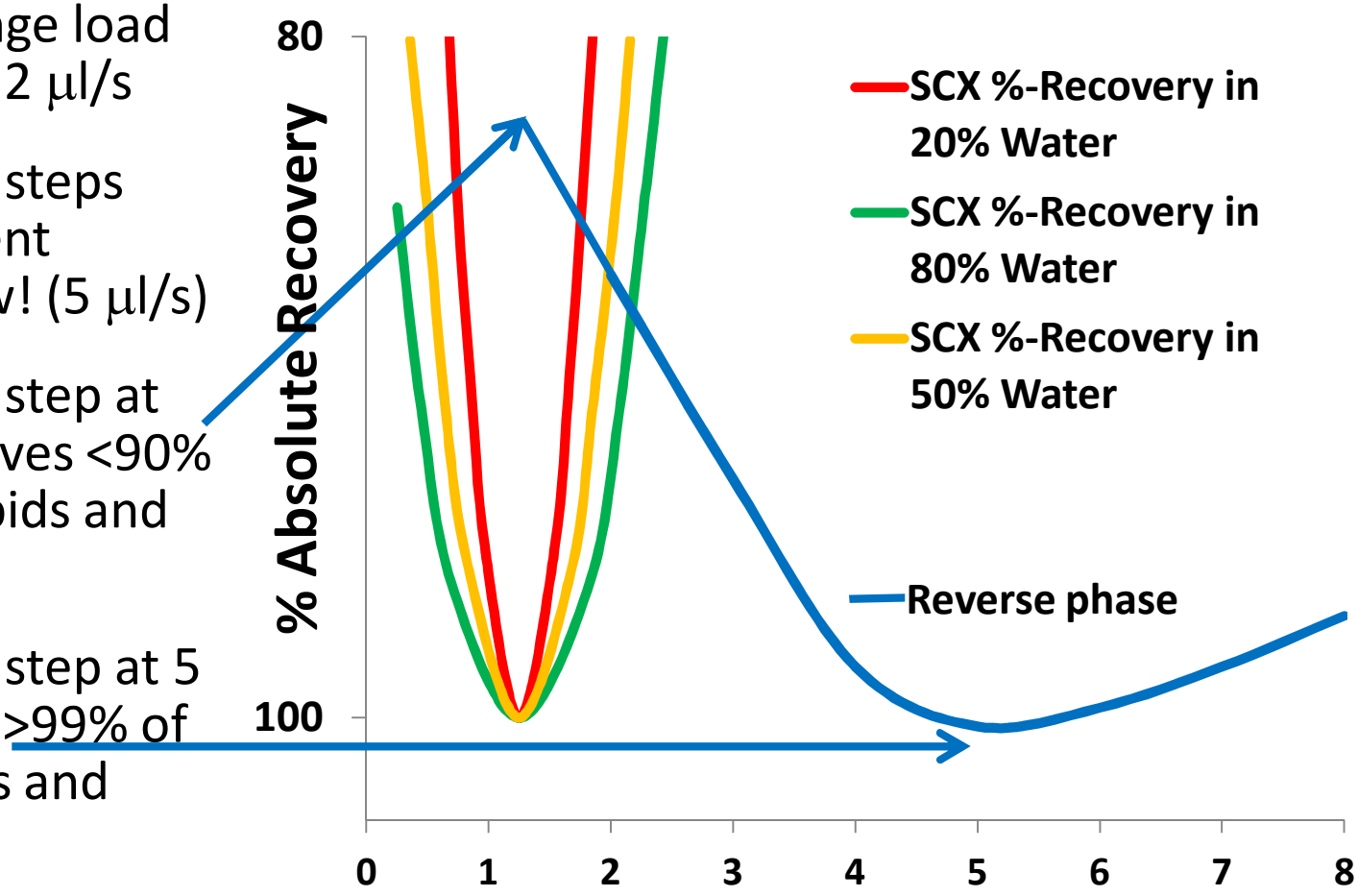
- Optimum flow for cation exchange load and elute = 1.2 μl/s
- Solvent wash steps have a different optimum flow! (5 μl/s)
- Solvent wash step at 1.2 μl/s removes <90% of phospholipids and fatty acids
- Solvent wash step at 5 μl/s removes >99% of phospholipids and fatty acids



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

SmartSPE: Basic Drugs in Blood

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

- Sample: 100 μl, 2/1 IPA crash, centrifuge, load 150 μl, 100 μl elute (H_2O/IPA/NH_4OH)
- Washes: 0.02M pH 6 phosphate buffer (optional), IPA/MeOH, H_2O
- All cutoffs: ≤1 ng/ml (S/N =20+, up to 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 – 240/24hr)
- Removes:
  - Lipids: *phospholipids*, fatty acids, triglycerides, cholesterol, uncharged oils
  - Salts, oxidized & intact sugars, organic & amino acids
- >99% absolute recoveries (validated immunosuppressants & 63 abused drugs)
- 2D-LC (trap & elute) aft SPE 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

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SmartSPE: Basic Drugs in Blood

impact in forensics labs (goal: do more without costing more)

- Typical forensics lab is performing SPE manually, which limits throughput to 24 samples per day per technologist
- A CTC/PAL (MPS) can SPE 240 samples per day
- Typical actual outcomes in forensics labs:
  - 4x increase in capacity with same technologists
  - Cleaner samples results in less maintenance issues (not more due to more samples)
  - Quality: QC and proficiency samples are generally 100% ID and observed within ±3% of actual concentration
  - Credibility: methods can be validated against external standards (no matrix effects) and chain of custody can be made bulletproof with addition of bar code reader to PAL (MPS)
- In addition, a CTC/PAL (MPS) often can be purchased via a state/federal law enforcement block grant resulting in zero impact on local operating/capital budgets

Summary and Conclusions

- **With flow control and packed sorbent, SPE is gradient LC**
  - Knowledge about gradient LC can be applied to SPE
  - Applying this knowledge produces significantly better results than alternatives (systematic 99+% recovery/matrix removal)
- **Adsorption and desorption is a reversible equilibrium**
  - Separate measurement of load and elute flow behavior produces the same van Deemter curves
  - Key chromatography assumption (C term: velocity on = velocity off) *empirically* shown to be true under gradient conditions
- **SPE with single use devices & accurate flow achieves high quality in high-throughput applications & provides high-productivity impact for clinical & forensics applications**

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*

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OpAns LLC
(Ken Lewis - clinical & forensics validation & use)

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2D-SmartSPE for proteomics and lipidomics

(potential approaches for omics sample fractionation)