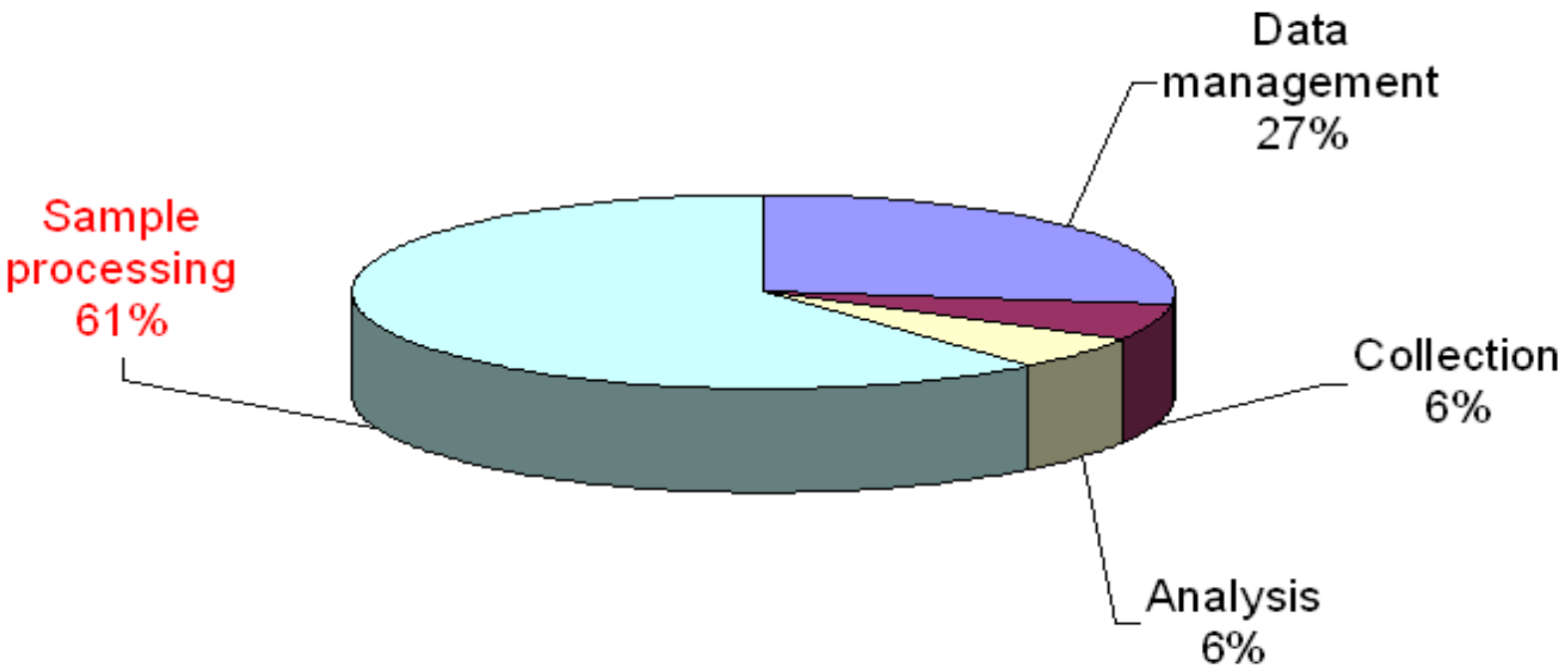
Sample Prep for Chromatography

Sorbents, Devices and Techniques to Improve Sensitivity, Specificity and Throughput

2010 Innovation Seminar Series
Supelco, Div. of Sigma-Aldrich

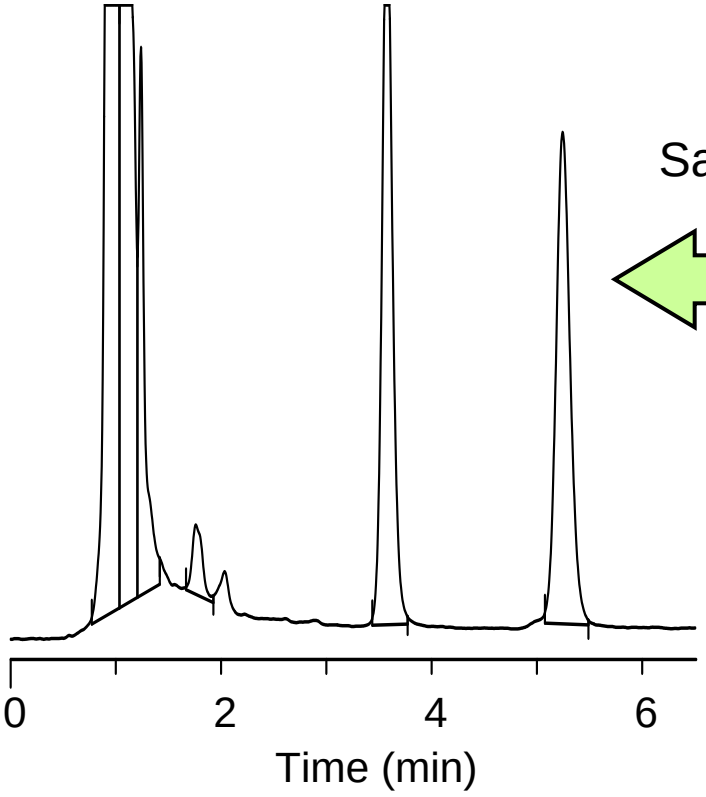
Time Spend on the Analytical Process



R.E. Majors, LC/GC Magazine, 1992, 1997, 2002

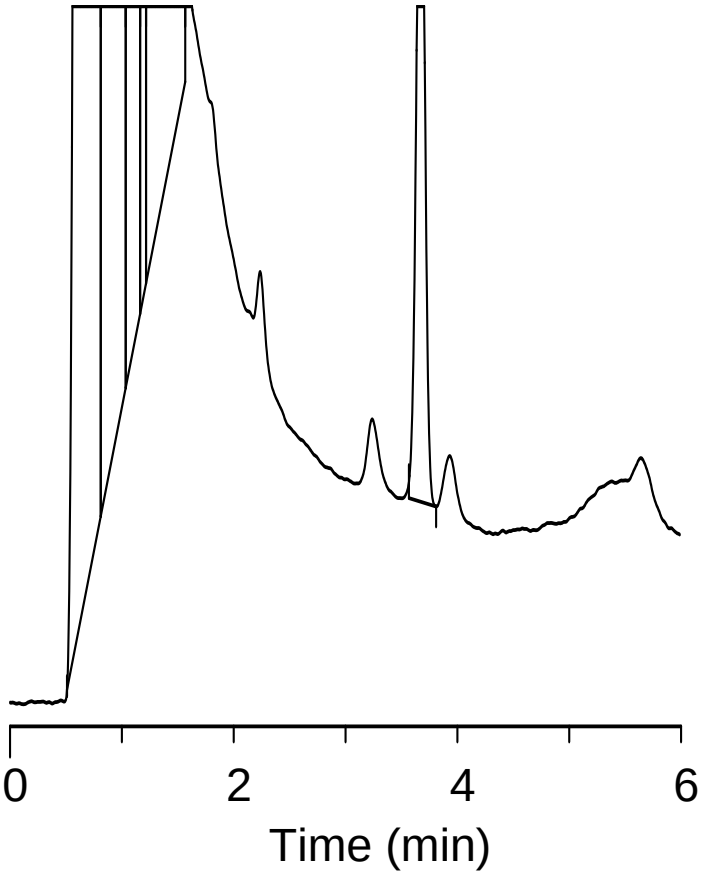
Real World & Real Samples

Ideal situation



Sample prep tools

Typical starting situation



Overview of Presentation

General SPE theory

Featured sample prep devices & techniques

1. Hybrid SPE particles
   - Improved sensitivity for LC-MS in bioanalysis
2. Molecularly-imprinted particles
   - Analyte/matrix-specific sorbents
3. Solid phase microextraction fibers
   - Solventless sample prep

For each device: What it is, how it works, examples

Goals of Sample Preparation

Remove matrix

Increase concentration of analyte

Exchange solvent

Other considerations:

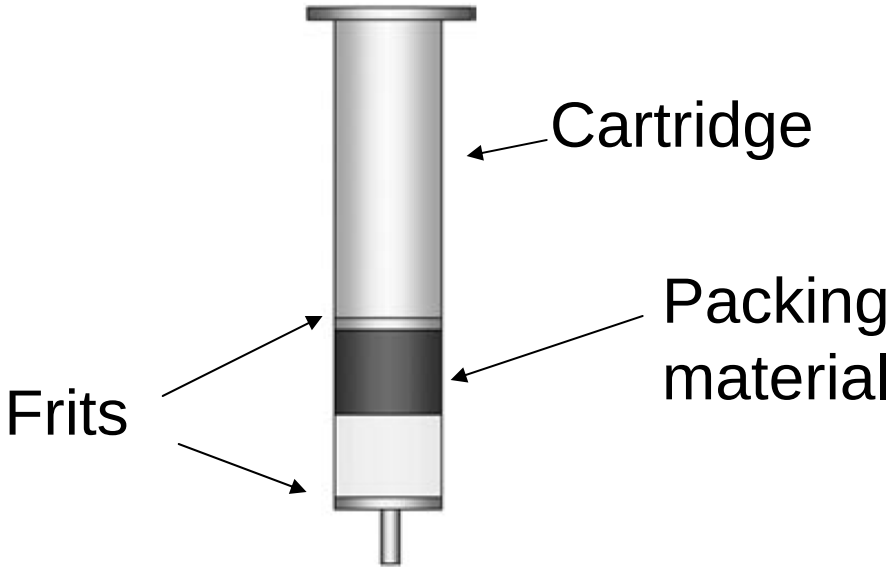
- Automation
- Exhaustive vs. equilibrium

Surface chemistry

- Affects k and alpha

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{k}{k+1} \cdot \frac{\alpha-1}{\alpha}$$

Basic SPE Concept



Three SPE Strategies

Bind-Elute Strategy

- Bind: Analytes bind to sorbent, unwanted matrix components are washed off
- Elute: Change eluant
- Analytes are concentrated via evaporation prior to analysis

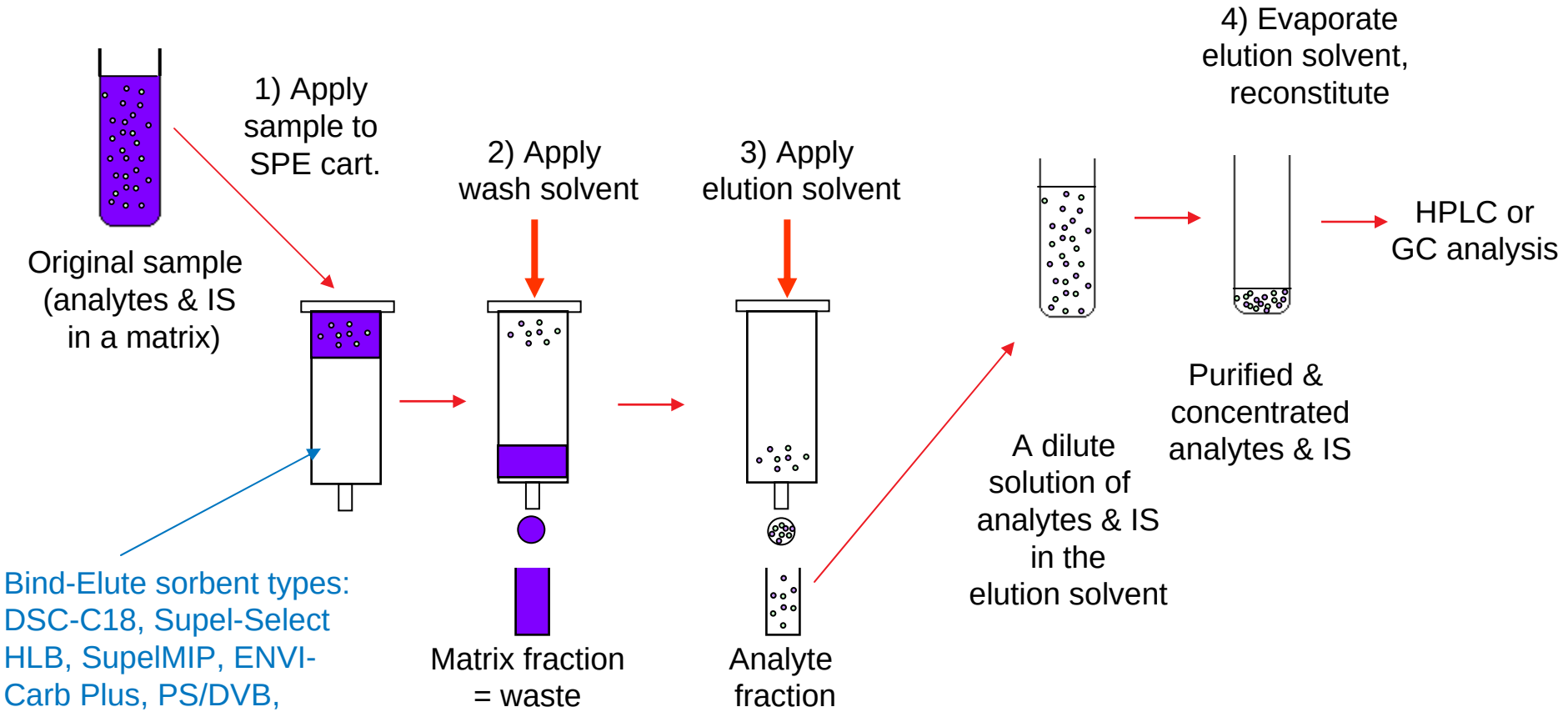
Interference Removal Strategy

- Bind all unwanted matrix components and allow analytes to pass through during the sample loading stage

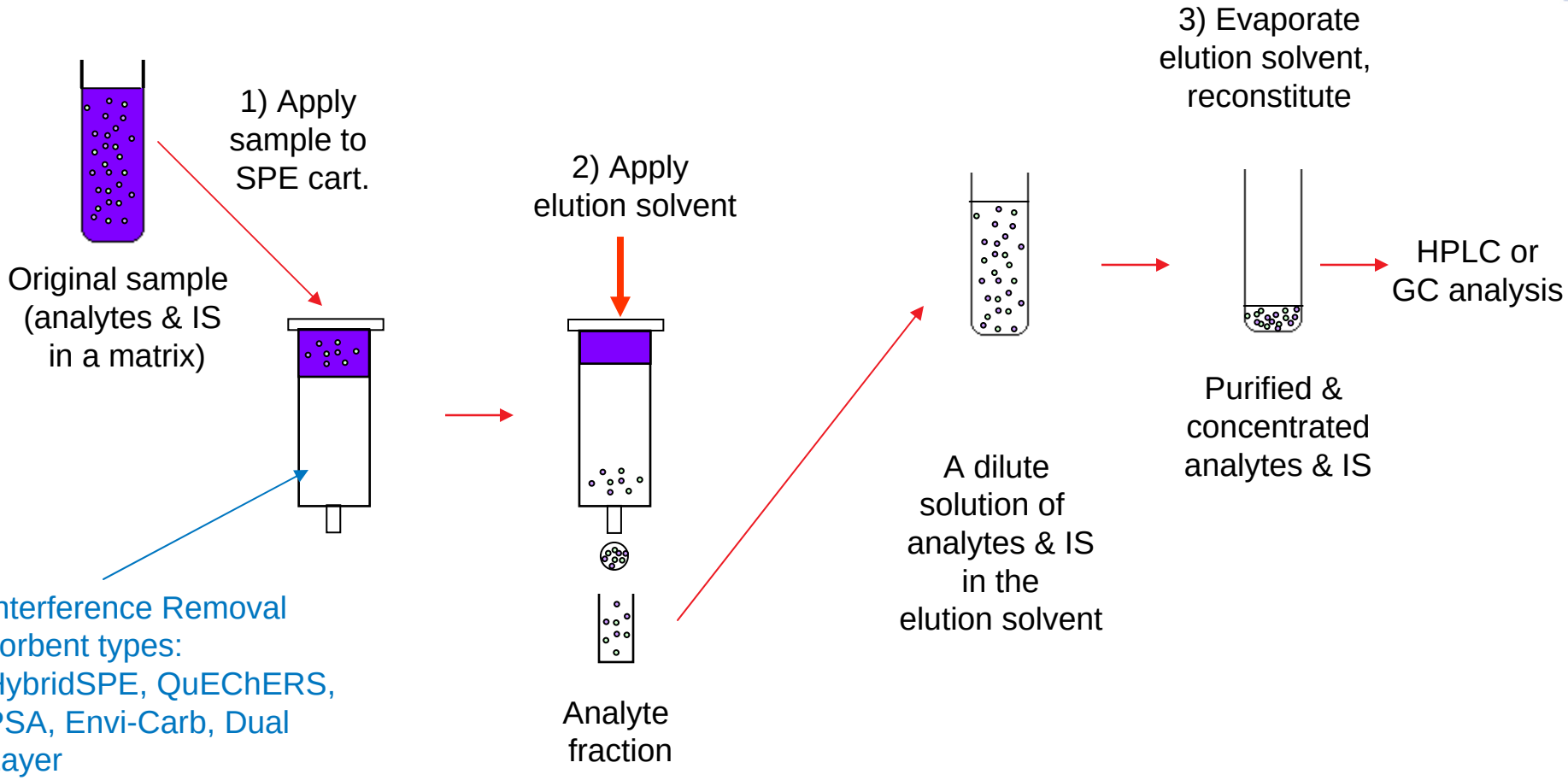
Fractionation Strategy

- Retain and sequentially elute different classes of compounds by modifying eluant pH or % organic

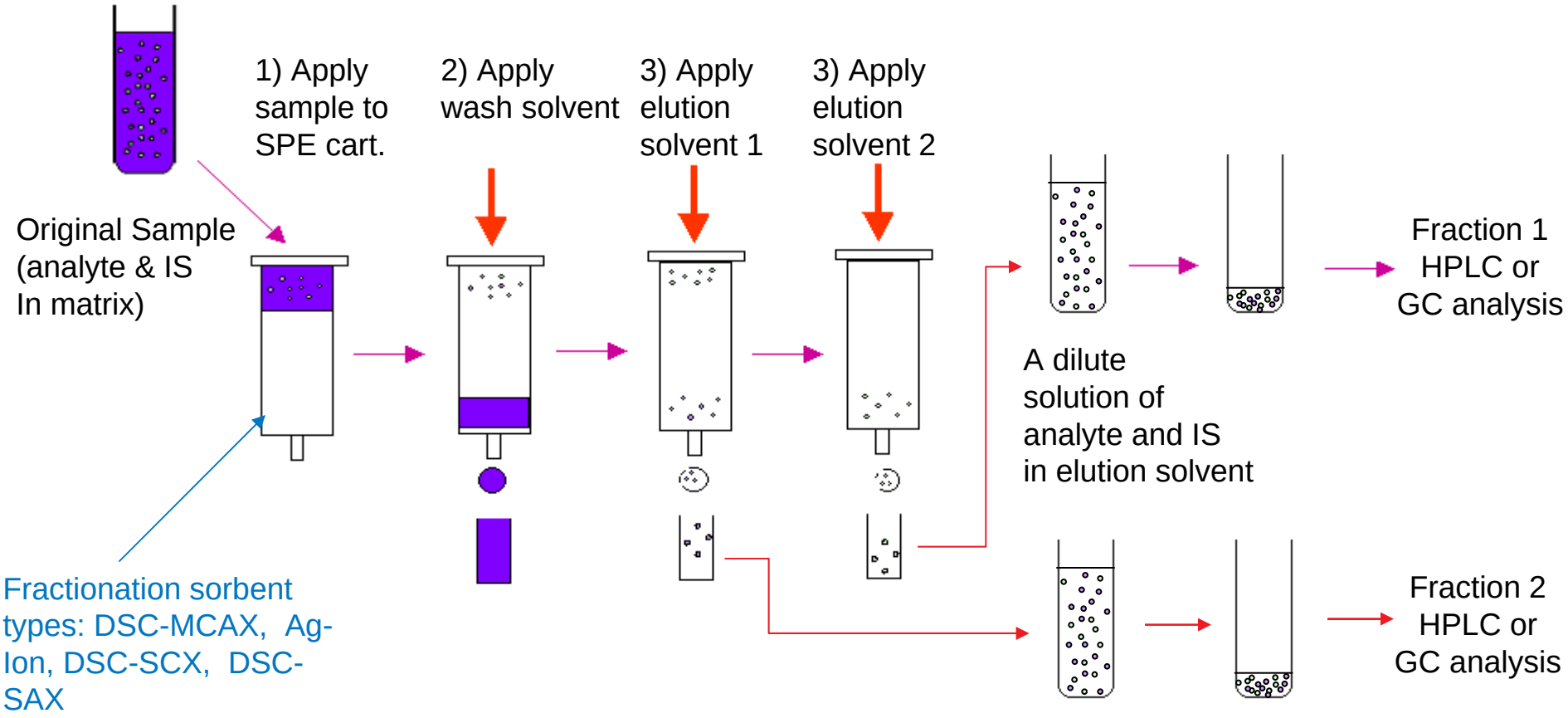
Bind-Elute Strategy Diagram



Interference Removal Strategy Diagram



Fractionation Strategy Diagram



Sample Prep Devices

Hybrid SPE particles

- Improved sensitivity for LC-MS in bioanalysis

Molecularly-imprinted particles

- Analyte/matrix-specific sorbents

Solid phase microextraction fibers

- Solventless sample prep

Strategy: Interference removal
Goal: Remove matrix

HybridSPE™-Precipitation (HybridSPE-PPT)

96-well SPE plates and cartridges

Zirconia-coated silica particles

Features:

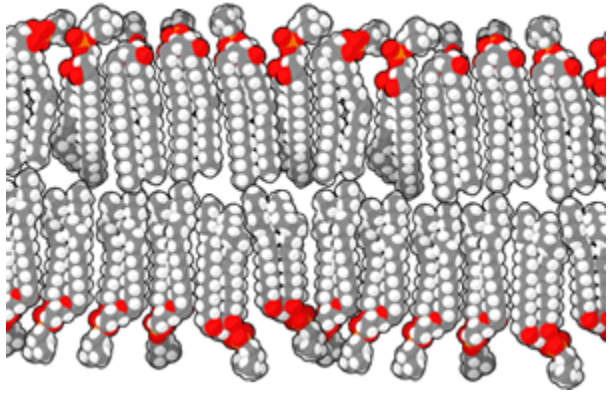
- Selective removal of phospholipid interferences and precipitated proteins
- Simple 2-3 step procedure

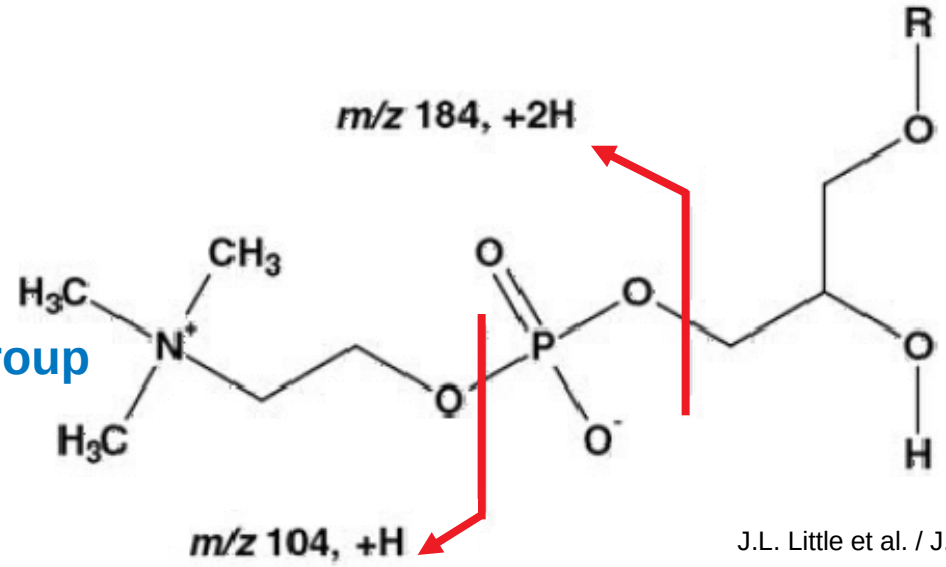
Benefits

- Improved LC-MS sensitivity (reduced matrix effect)
- Enhanced column lifetime
- Gradients not needed to clean column

Monitoring Phospholipid Contamination

- PLs major component of cell membranes
- Polar head group, non-polar tail
- Largest subclass (phosphatidylcholine) monitored using m/z 184 or m/z 104 fragment ions
- Used as a marker for ion-suppression risk assessment during LC-MS/MS
- Determine selectivity effectiveness of sample prep technique





J.L. Little et al. / J. Chromatogr. B 833 (2006) 219–230

Problem: Protein and Phospholipid Accumulation on HPLC Column

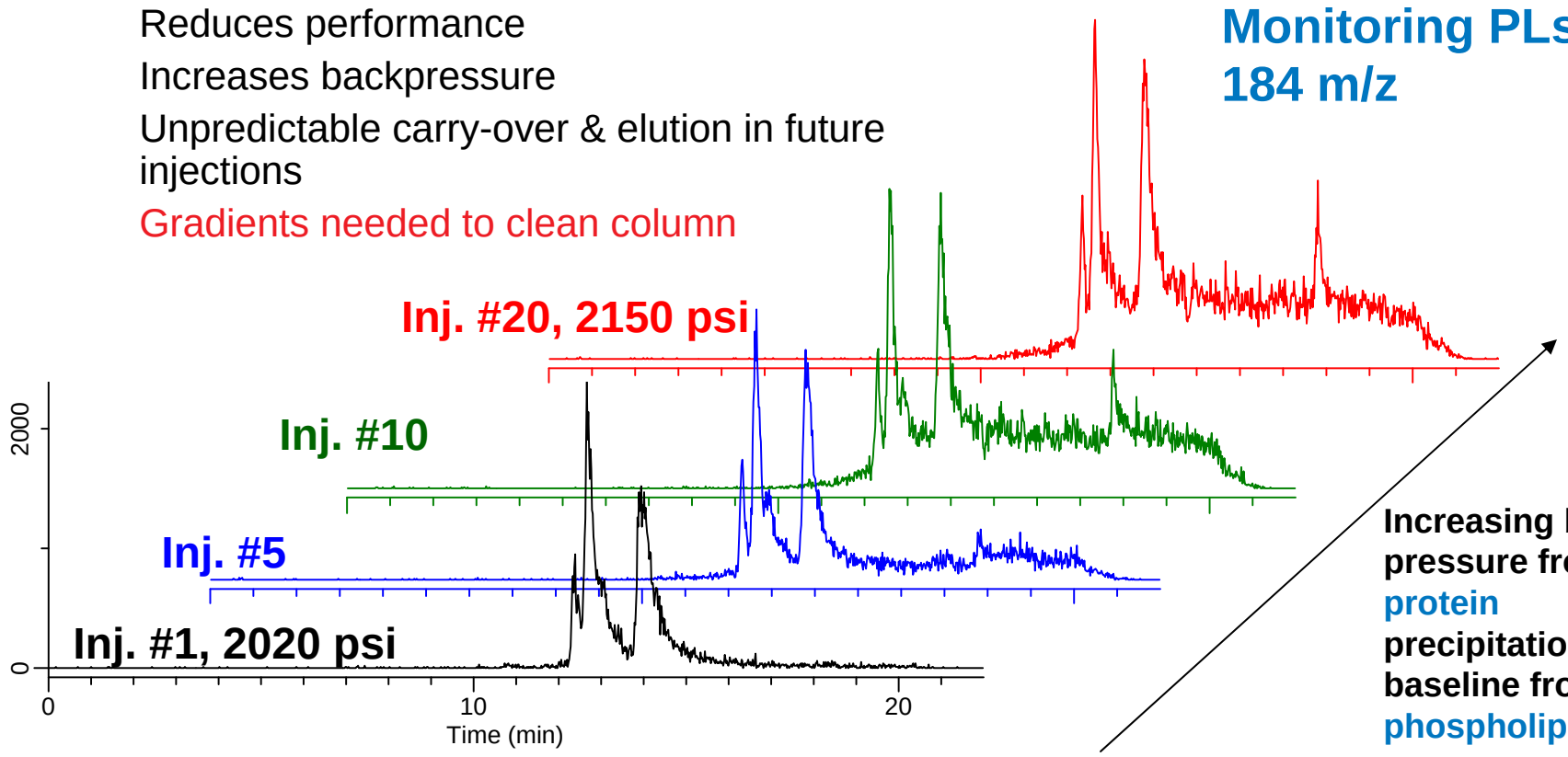
Standard protein ppt technique

Reduces performance

Increases backpressure

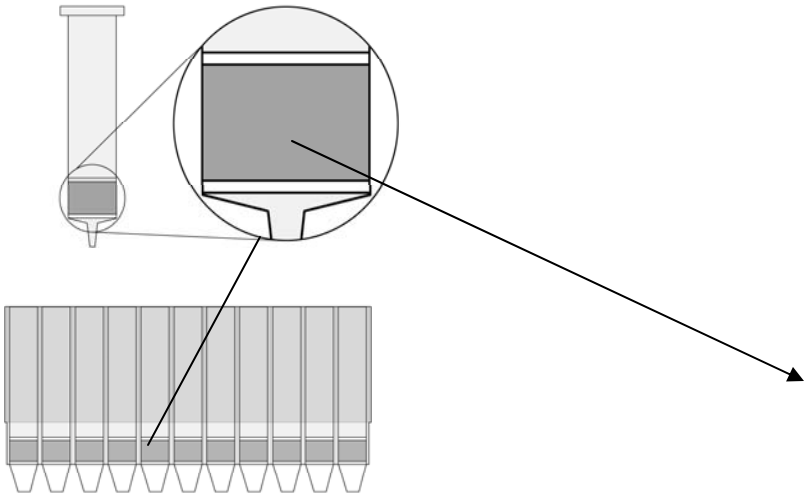
Unpredictable carry-over & elution in future injections

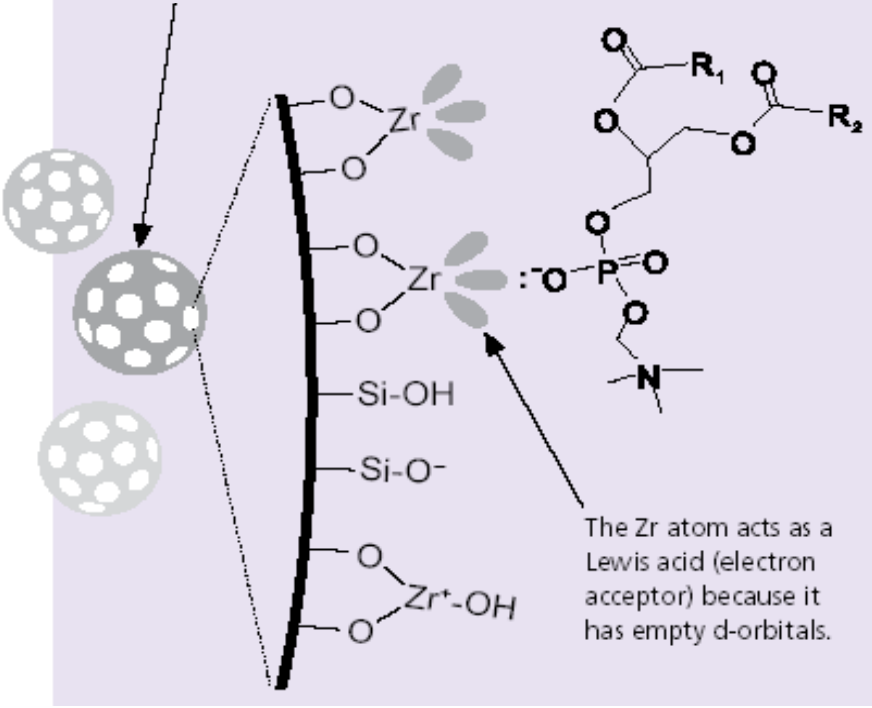
Gradients needed to clean column



14 **HPLC column:** Sub-2um C18, 5 cm x 2.1 mm I.D.

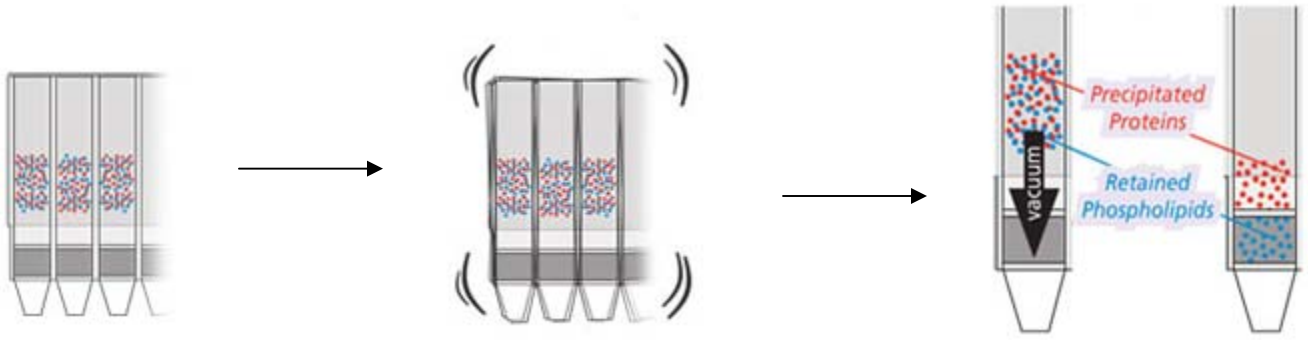
Solution: Phospholipids Selectively Removed using HybridSPE-PPT Technology





- The Zr atom on the particle acts as a Lewis acid
- The phosphate groups on the phospholipids are strong Lewis bases and complex with the zirconium atoms
- Analytes are eluted free of phospholipids[15]

HybridSPE-PPT Method (96-Well Format)

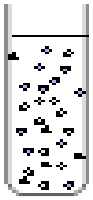


Precipitate proteins in well

Mix

Apply vacuum

- 100 µL plasma/serum
- 300 µL 1% formic acid in acetonitrile
- Add I.S. as necessary



Resulting filtrate/eluate is free of proteins and phospholipids, ready for LC-MS

Improved Situation: No Protein or Phospholipid Accumulation Using HybridSPE-PPT

Consistent column performance

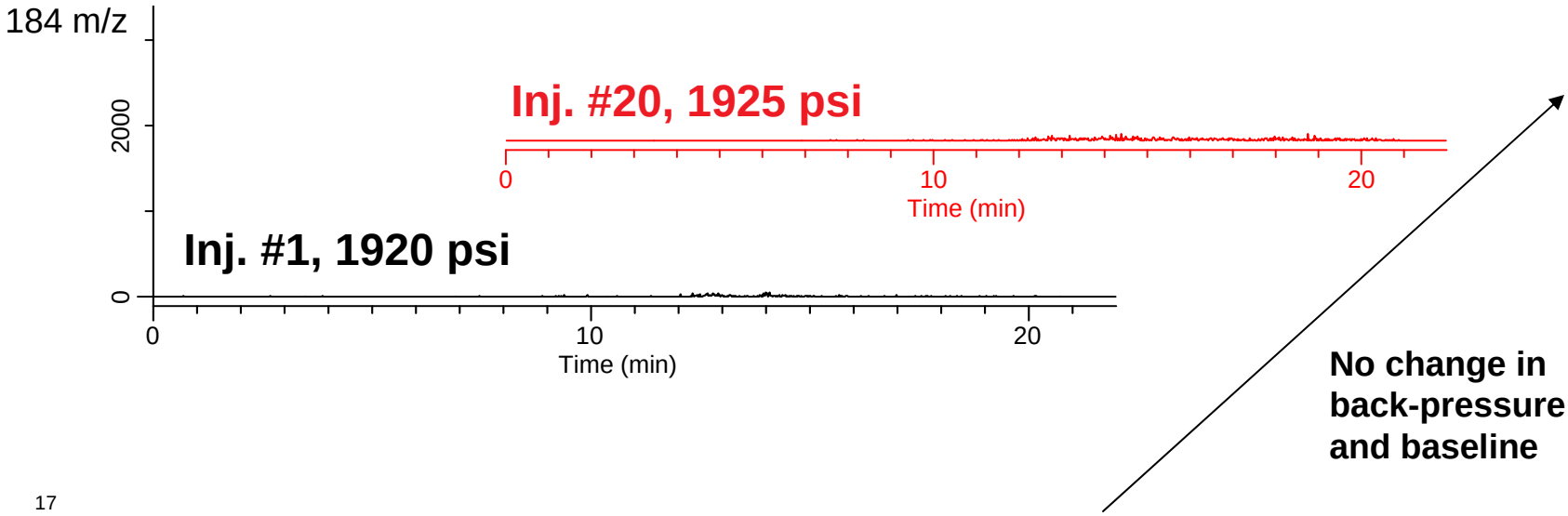
No increase in backpressure

Eliminates carry-over & elution in future injections

Extends column lifetime

Gradients are not needed to clean column

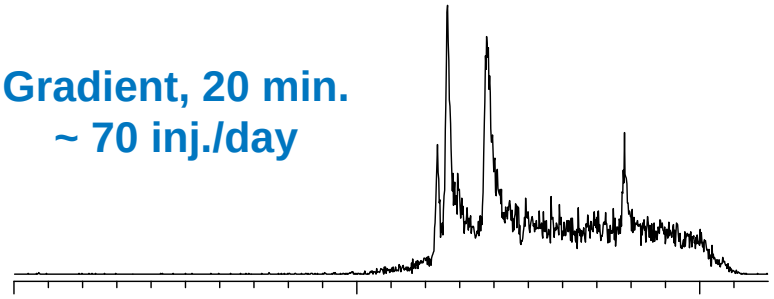
Monitoring PLs at 184 m/z



Improved Through-put with HybridSPE-PPT

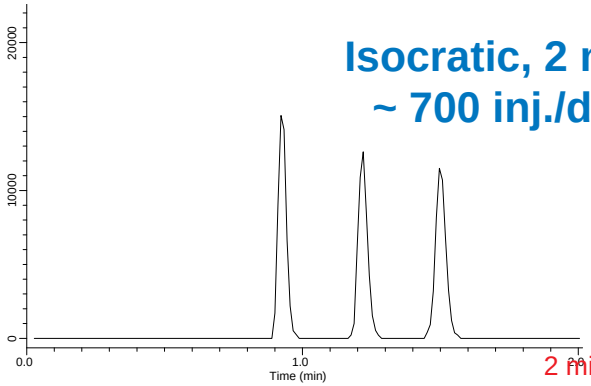
Elimination of need for gradient clean-up improves sample throughput

Gradient, 20 min.
~ 70 inj./day

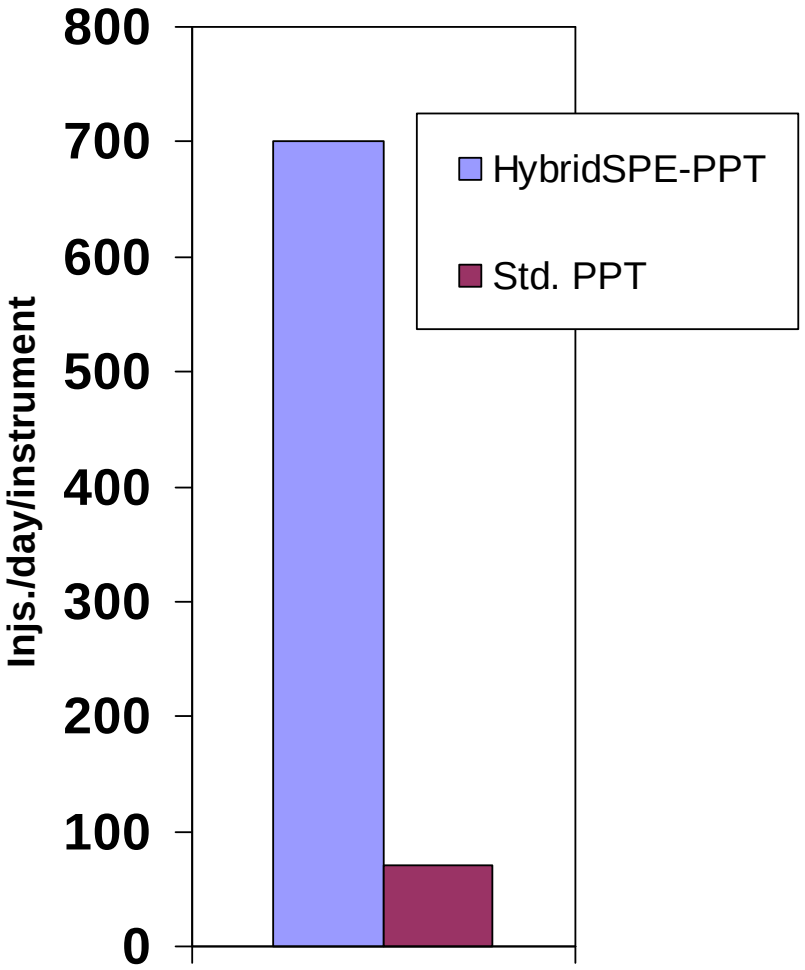


20 min.

Isocratic, 2 min.
~ 700 inj./day



2 min.

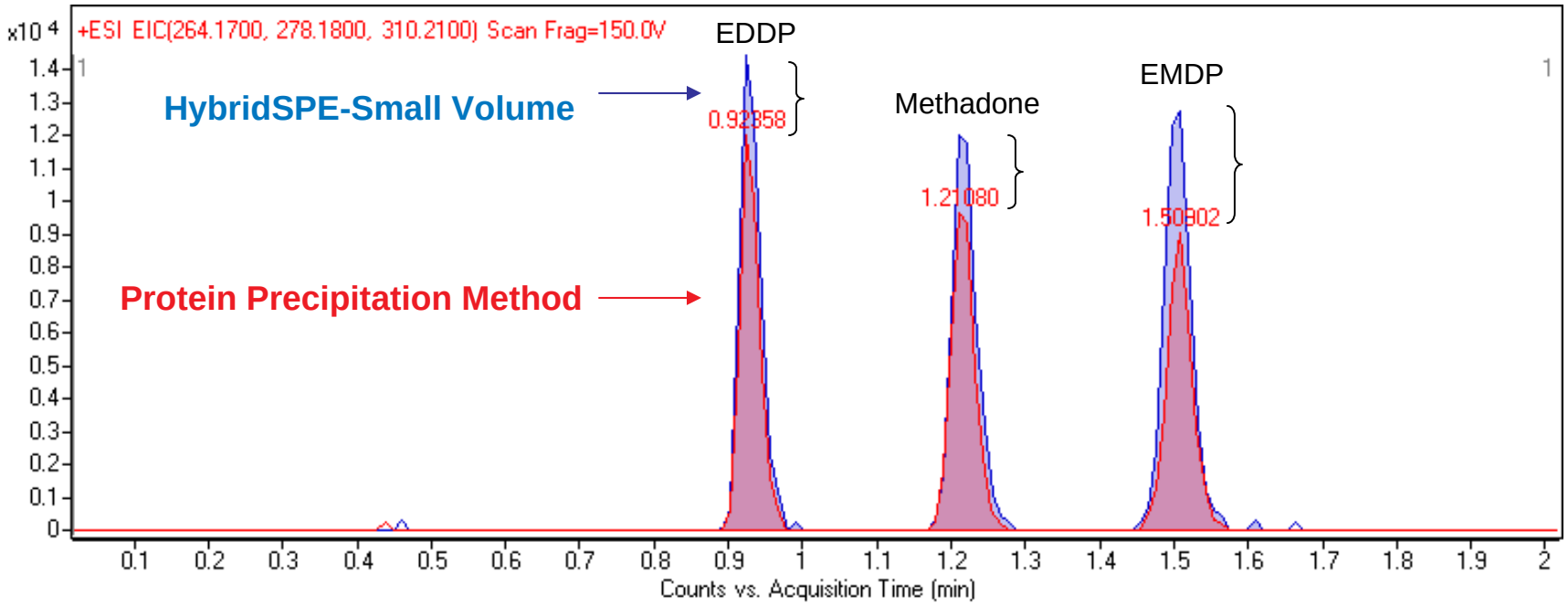


Overlay of HybridSPE-Small Volume and Protein Precipitation Samples

Methadone and metabolites from plasma

Sample was extracted using HybridSPE-PPT or standard PPT

High concentration (1200 ng/mL), still shows suppression with standard ppt method



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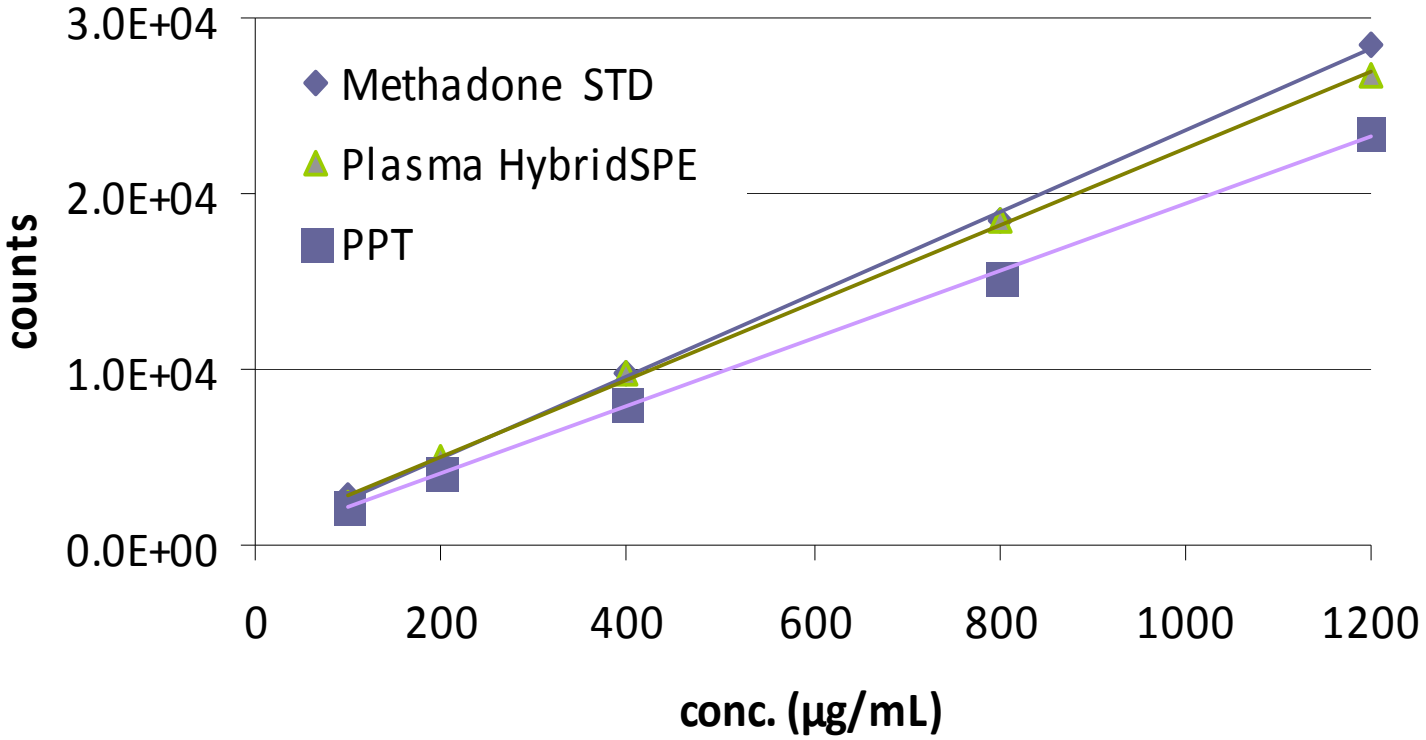
Column: Ascentis Express RP-Amide 10 cm X 2.1, mm I.D., 2.7um; ESI+ detection

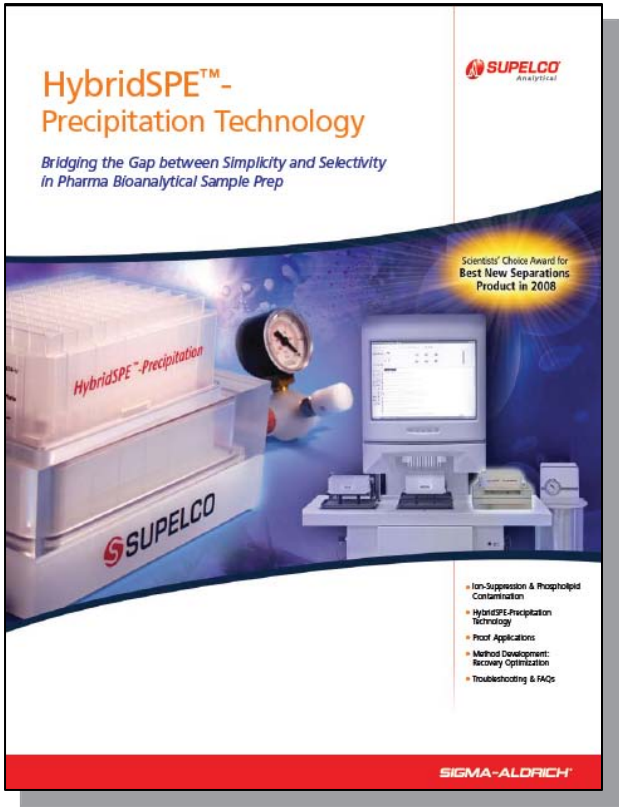
Calibration: Methadone Plasma Extracts

Comparison of response HybridSPE-PPT vs. standard protein ppt



HybridSPE™ – Precipitation Technology (HybridSPE-PPT)

- Simplicity of protein precipitation and selectivity of SPE
- Nearly complete depletion of phospholipids and precipitated proteins
- 2-3 step generic procedure
- 96-well and cartridge dimensions
  - 50 mg/2 mL per well
  - 15 mg/0.8 mL per well
- Compatible with automation
- No need for gradients to clean HPLC column



Sample Prep Devices

Hybrid SPE particles

- Improved sensitivity for LC-MS in bioanalysis

Molecularly-imprinted particles

- Analyte/matrix-specific sorbents

Solid phase microextraction fibers

- Solventless sample prep

Strategy: Bind-elute
Goal: Remove matrix

High-Specificity SPE (SupelMIPs)

96-well SPE plates and cartridges

Molecularly imprinted polymer particles

Developed by MIP Technologies, Lund, Sweden

Features:

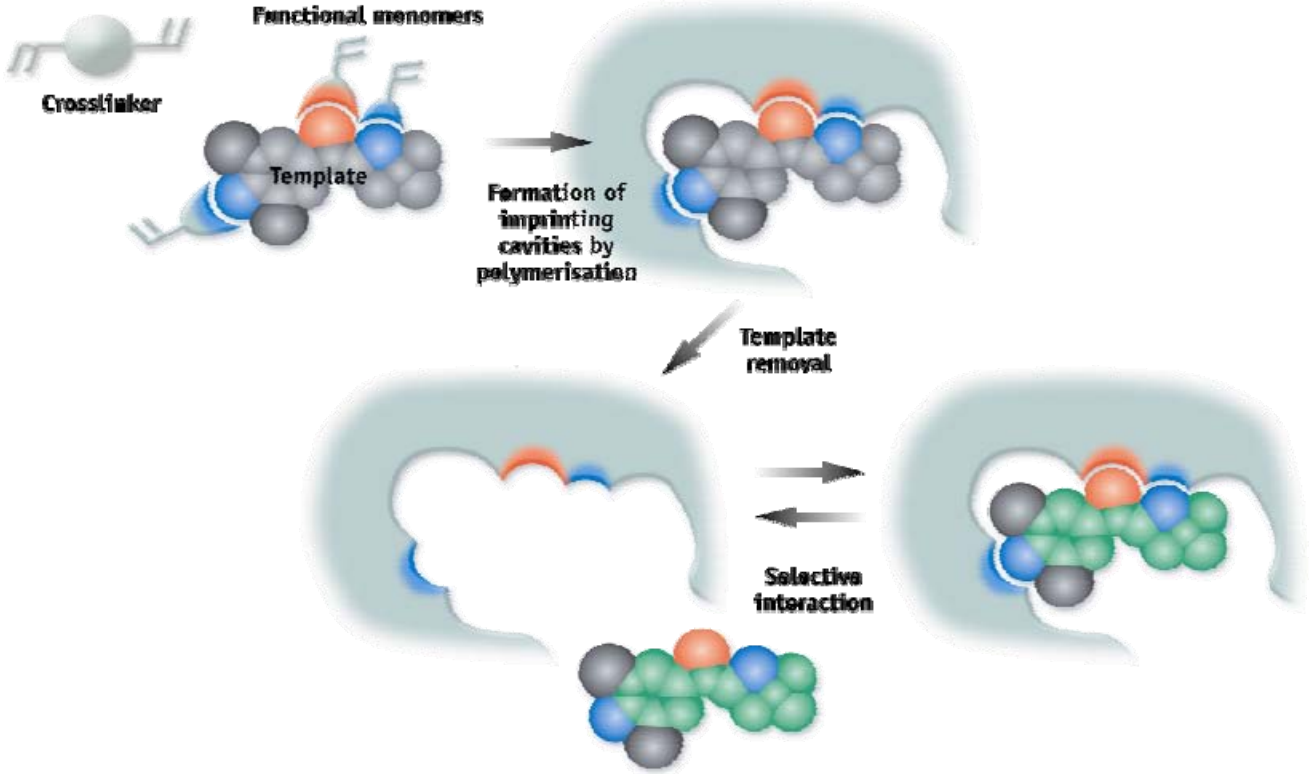
- Very selective extraction
- Predefined protocols: no method development

Benefits:

- Permits more rigorous washing to remove matrix
- Analysis at extremely low concentrations (ppb, ppt)

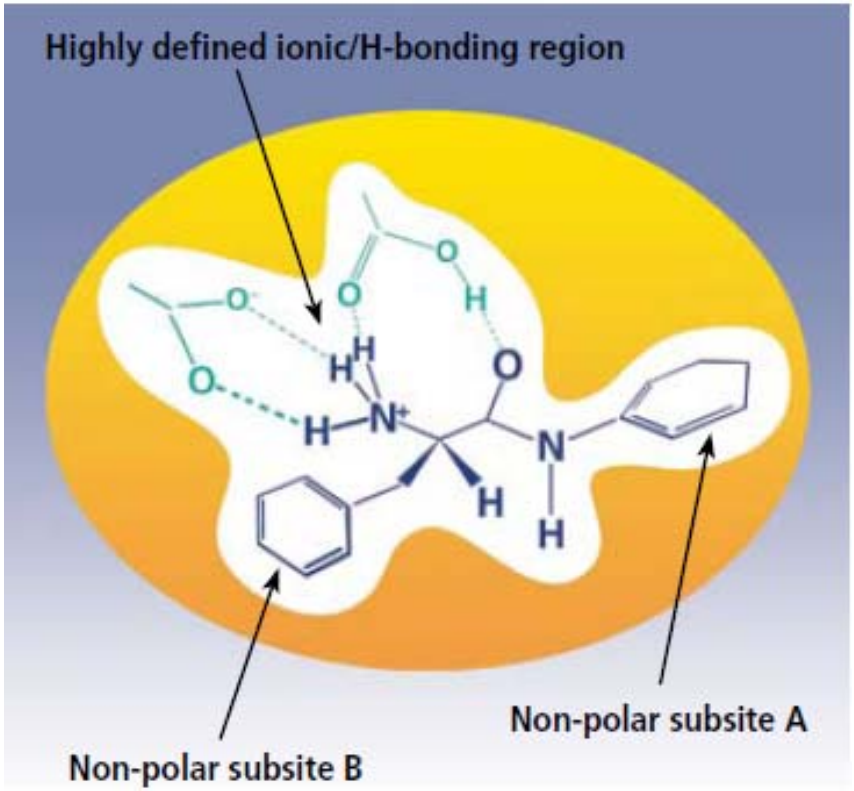
The Molecular Imprinting Process

Molecularly imprinted polymers (MIPs) are polymers that have been prepared by polymerizing either pre-formed or self-assembled monomer-template complexes together with a cross-linking monomer. After removal of the template molecule, a polymer with binding sites for the template is obtained.



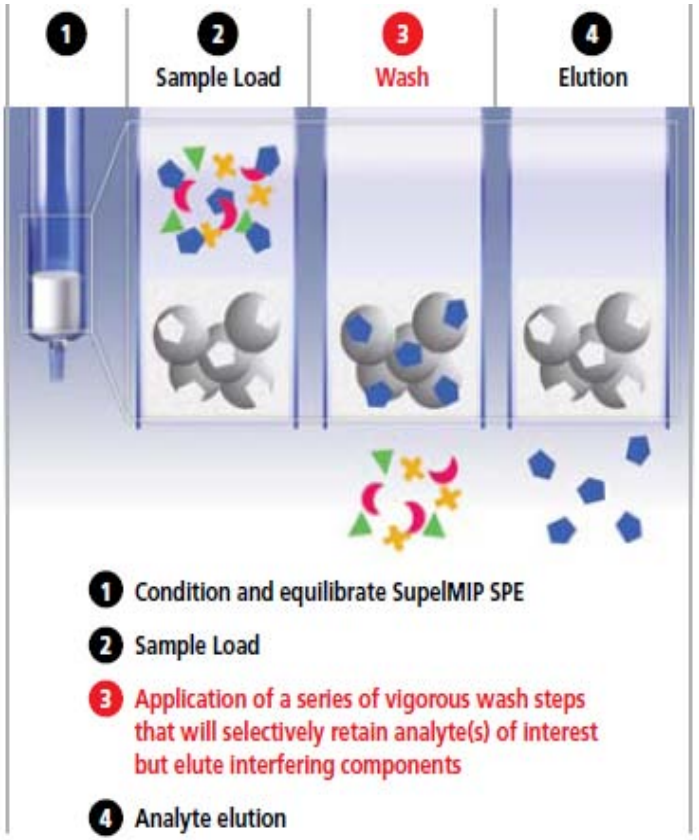
The MIP Binding Site

Graphical representation of the MIP binding site, which contains a cavity of the right size and attractive molecular features that can bind to the target molecule(s).



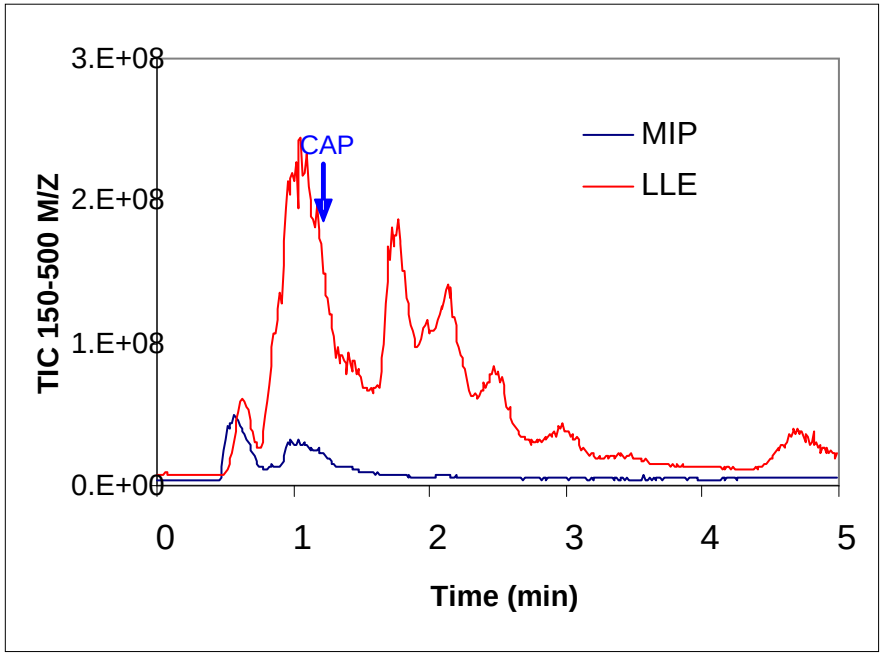
Overview of a Typical SupelMIP SPE Procedure

- Very simple methods.
- Full protocols are included with each MIP product.
- Protocols may require optimization depending on the sample matrix.

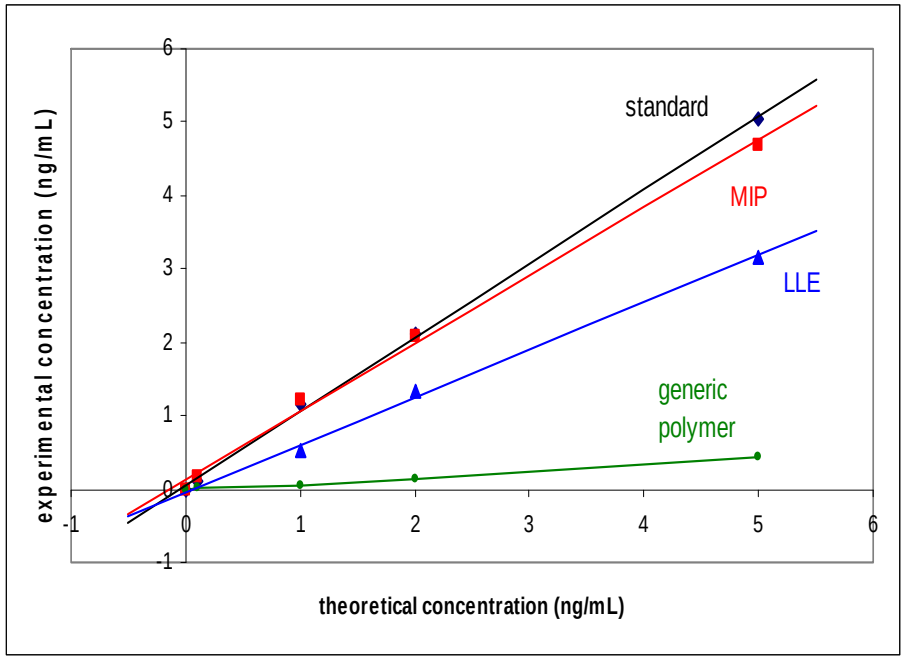


SupelMIP Chloramphenicol: Analysis in Honey

Chloramphenicol is an antibiotic that is monitored in honey.



Background from honey sample cleaned by SupelMIP-SPE and LLE for chloramphenicol analysis.



Comparison of matrix effect (ion suppression) between different clean-up methods for honey. Samples were spiked with CAP prior to analysis.

SupelMIP Products

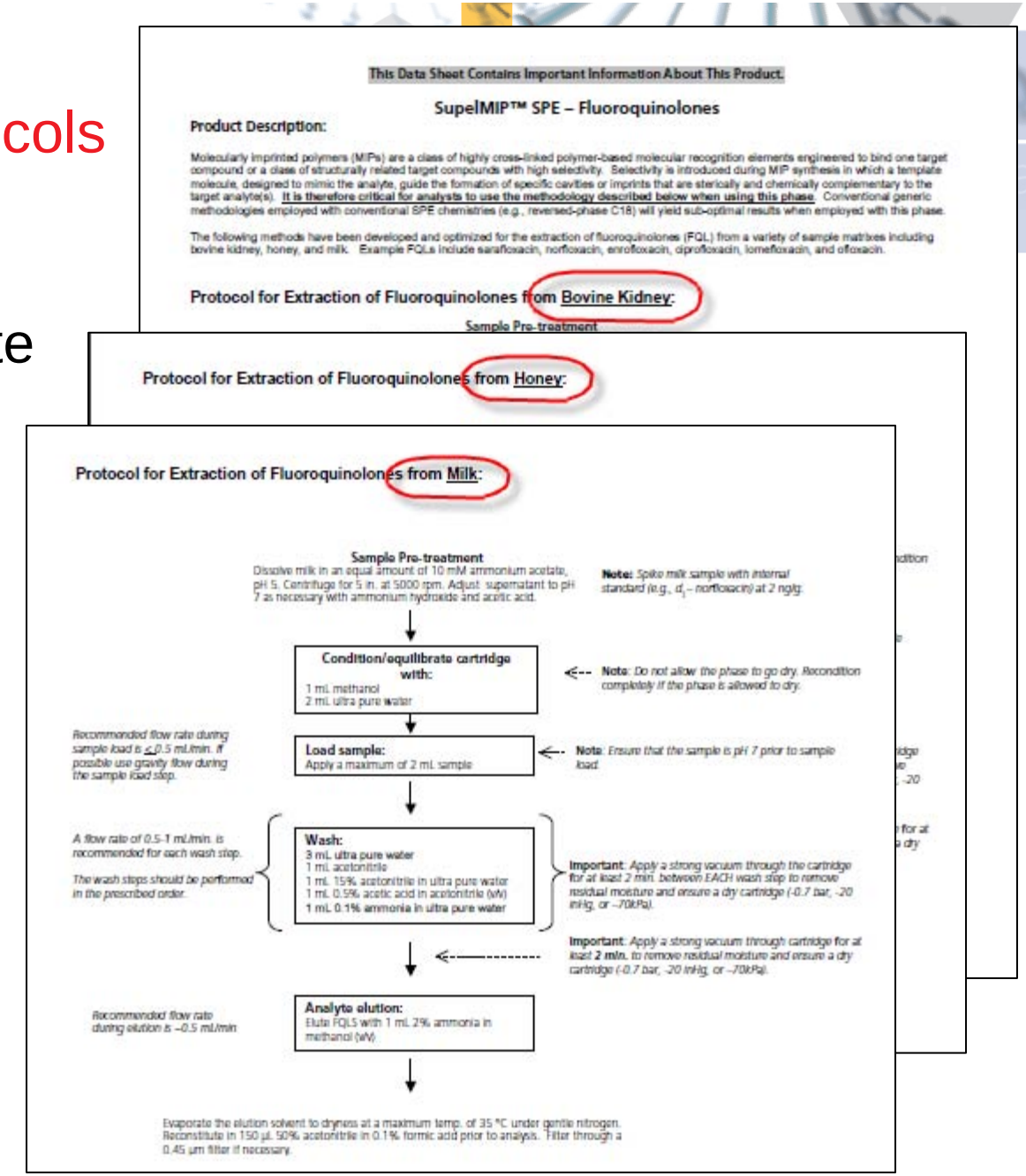
- **PAHs** in edible oils
- **Nitroimidazoles** in milk, eggs and other foods
- Nonsteroidal anti-inflammatory drugs (**NSAIDS**) in wastewater and other matrices
- **Fluoroquinolones** in bovine kidney, honey and milk
- **Amphetamines** and related compounds in urine
- **Chloramphenicol** in plasma, urine, milk, honey and shrimp
- **NNAL** - nitroso compound in urine
- **TSNAs** - tobacco specific nitrosamines in urine and tobacco
- **β-agonists** and **β-blockers** in tissue, urine and wastewater
- **Clenbuterol** in urine
- **Triazines** in water
- **Riboflavin** in milk

In development:

- **Nicotine** and **Cotinine** in gum, urine
- **Aminoglycosides** in cell culture broth, honey, kidney
- **Crystal violet** in fish tissue
- **Malachite green** in fish tissue

Detailed MIP Protocols

- Optimized for analyte and matrix
- Eliminates method development time



This Data Sheet Contains Important Information About This Product.

SupelMIP™ SPE – Fluoroquinolones

Product Description:

Molecularly imprinted polymers (MIPs) are a class of highly cross-linked polymer-based molecular recognition elements engineered to bind one target compound or a class of structurally related target compounds with high selectivity. Selectivity is introduced during MIP synthesis in which a template molecule, designed to mimic the analyte, guide the formation of specific cavities or imprints that are sterically and chemically complementary to the target analyte(s). **It is therefore critical for analysts to use the methodology described below when using this phase.** Conventional generic methodologies employed with conventional SPE chemistries (e.g., reversed-phase C18) will yield sub-optimal results when employed with this phase.

The following methods have been developed and optimized for the extraction of fluoroquinolones (FQL) from a variety of sample matrixes including bovine kidney, honey, and milk. Example FQLs include sarafloxacin, norfloxacin, enrofloxacin, ciprofloxacin, lomefloxacin, and ofloxacin.

Protocol for Extraction of Fluoroquinolones from Bovine Kidney:

Sample Pre-treatment

Protocol for Extraction of Fluoroquinolones from Honey:

Protocol for Extraction of Fluoroquinolones from Milk:

Sample Pre-treatment
Dissolve milk in an equal amount of 10 mM ammonium acetate, pH 5. Centrifuge for 5 in. at 5000 rpm. Adjust supernatant to pH 7 as necessary with ammonium hydroxide and acetic acid.

Note: *Spike milk sample with internal standard (e.g., d_5 – norfloxacin) at 2 ng/g.*

Condition/equilibrate cartridge with:
1 mL methanol
2 mL ultra pure water

Note: *Do not allow the phase to go dry. Recondition completely if the phase is allowed to dry.*

Recommended flow rate during sample load is ≤0.5 mL/min. If possible use gravity flow during the sample load step.

Load sample:
Apply a maximum of 2 mL sample

Note: *Ensure that the sample is pH 7 prior to sample load.*

A flow rate of 0.5-1 mL/min. is recommended for each wash step.

The wash steps should be performed in the prescribed order.

Wash:
3 mL ultra pure water
1 mL acetonitrile
1 mL 15% acetonitrile in ultra pure water
1 mL 0.5% acetic acid in acetonitrile (v/v)
1 mL 0.1% ammonia in ultra pure water

Important: *Apply a strong vacuum through the cartridge for at least 2 min. between EACH wash step to remove residual moisture and ensure a dry cartridge (-0.7 bar, -20 inHg, or –70kPa).*

Important: *Apply a strong vacuum through cartridge for at least **2 min.** to remove residual moisture and ensure a dry cartridge (-0.7 bar, -20 inHg, or –70kPa).*

Recommended flow rate during elution is –0.5 mL/min

Analyte elution:
Elute FQLS with 1 mL 2% ammonia in methanol (v/v)

Evaporate the elution solvent to dryness at a maximum temp. of 35 °C under gentle nitrogen. Reconstitute in 150 µL 50% acetonitrile in 0.1% formic acid prior to analysis. Filter through a 0.45 µm filter if necessary.

Sample Prep Devices

Hybrid SPE particles

- Improved sensitivity for LC-MS in bioanalysis

Molecularly-imprinted particles

- Analyte/matrix-specific sorbents

Solid phase microextraction fibers

- Solventless sample prep, plus…

Strategy: Bind-elute
Goal: Analyte concentration

Solid Phase Microextraction (SPME)

Holder assemblies (manual, autosampler, robots)

Coated fibers (adsorbent and absorbent)

Janusz Pawliszyn, Univ. of Waterloo; unique and proprietary to Supelco

Economical enrichment technique mainly for trace analysis

Initially for GC analysis, now new fibers for LC

Features:

- Very limited or no use of solvents
- All types of samples & matrixes
- Direct immersion or headspace
- Designs for manual, autosamplers and robots

Benefits:

- Economical
- Highly consistent, quantifiable results
- Portable (field use) and reusable
- Reduces lab animal sacrifice

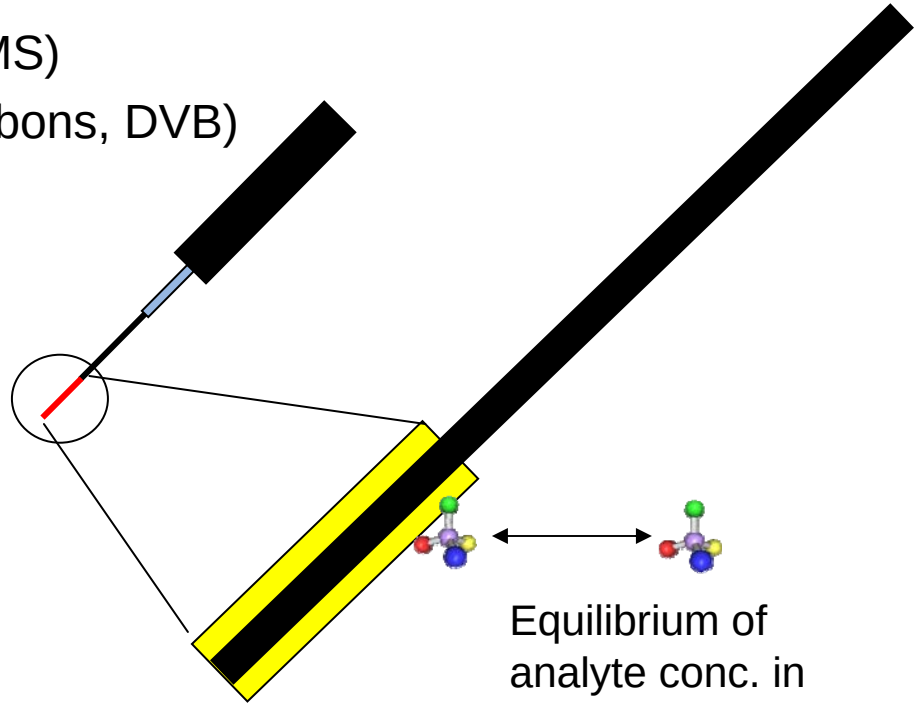
SPME Fiber Coating: The Business End

Not an exhaustive extraction technique

An equilibrium is set up between analytes dissolved in the sample (solution or gas phase) and in the liquid coating on the fiber.

The fiber coating consists of:

- Matrix/binder (e.g. PDMS)
- Particles (e.g. C18, carbons, DVB)

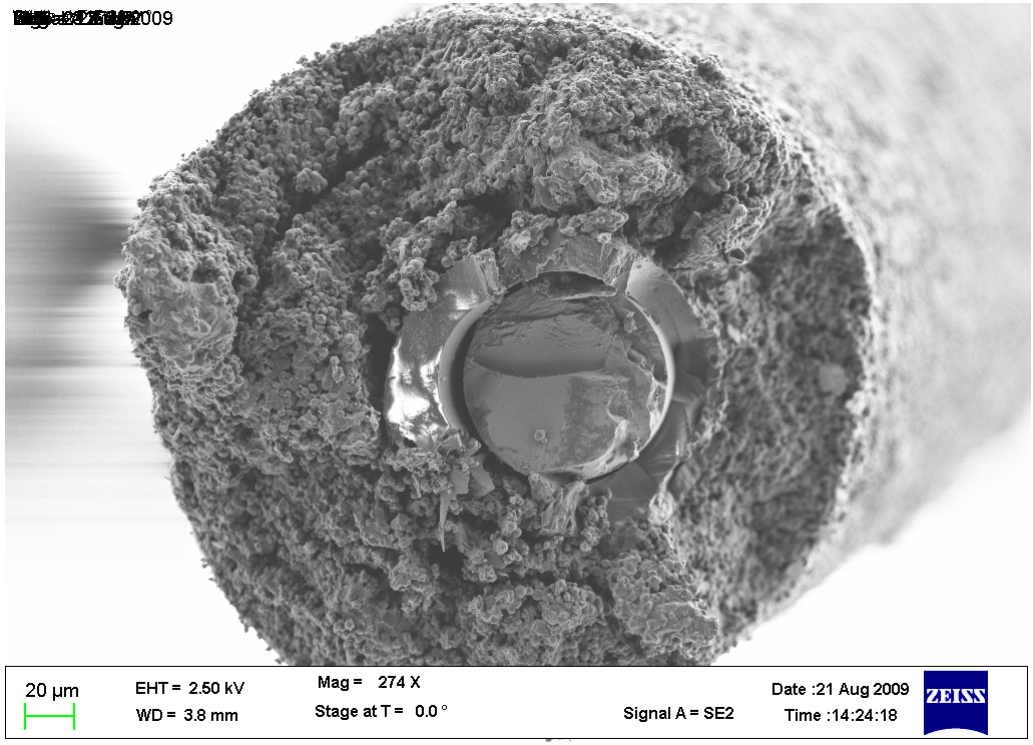


Enlargement of the SPME fiber coating

Equilibrium of analyte conc. in fiber and sample

PDMS-DVB Fiber SEM

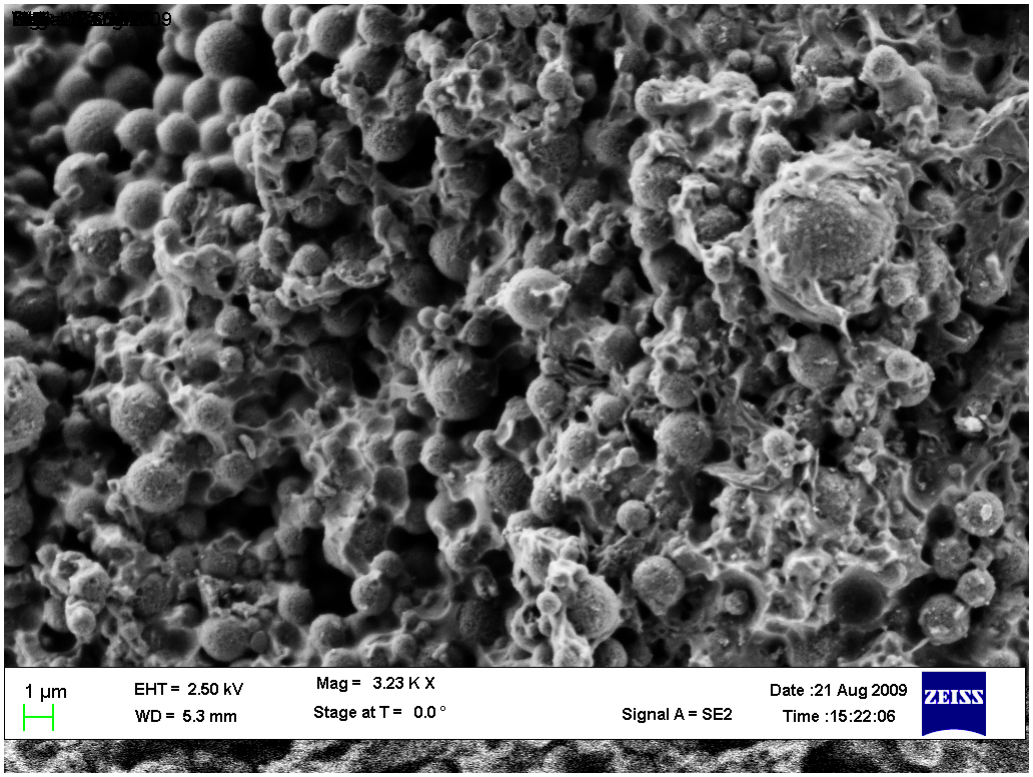
Cross section of the PDMS-DVB fiber. The center is a fused silica core, surrounded by a Stableflex core. The 3-5µm DVB particles are suspended in PDMS and layered over the cores. 275x magnification.



Photomicrograph of SPME fiber provided by Prof. Dan Armstrong, U. Texas Arlington

PDMS-Carboxen Fiber SEM

3000X magnification of the Carboxen PDMS coating. The 3-5µm Carboxen particles are suspended in PDMS.



Photomicrograph of SPME fiber provided by Prof. Dan Armstrong, U. Texas Arlington

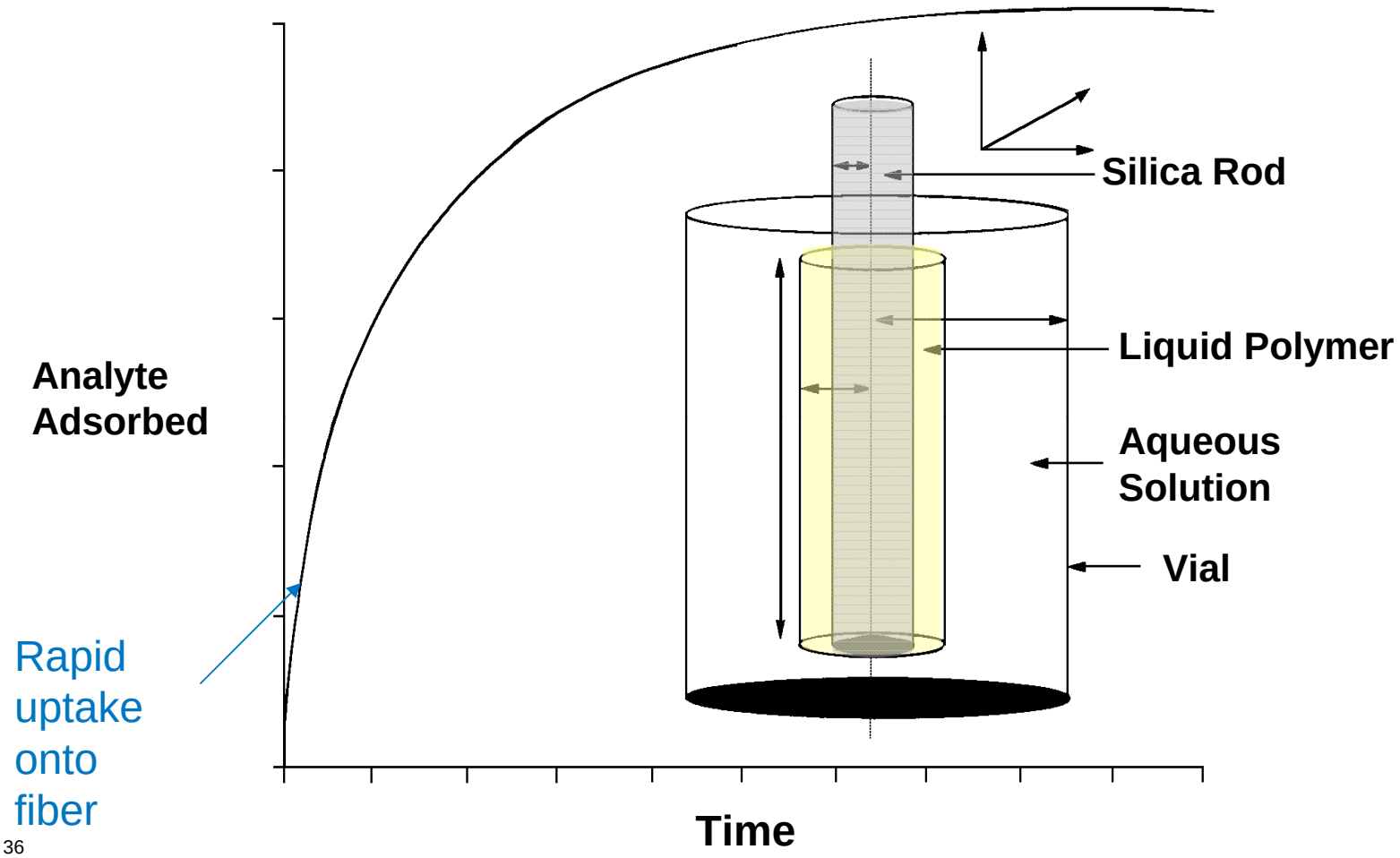
Distribution Constant

Concentration of analyte in stationary phase compared to concentration of analyte in solution:

$$K = n_s/V_1C_2^\circ$$

K = Distribution constant
n_s = Moles of analyte in stationary phase
V_1 = Volume of stationary phase
C_2° = Final analyte concentration in sample

Adsorption Mechanism for SPME

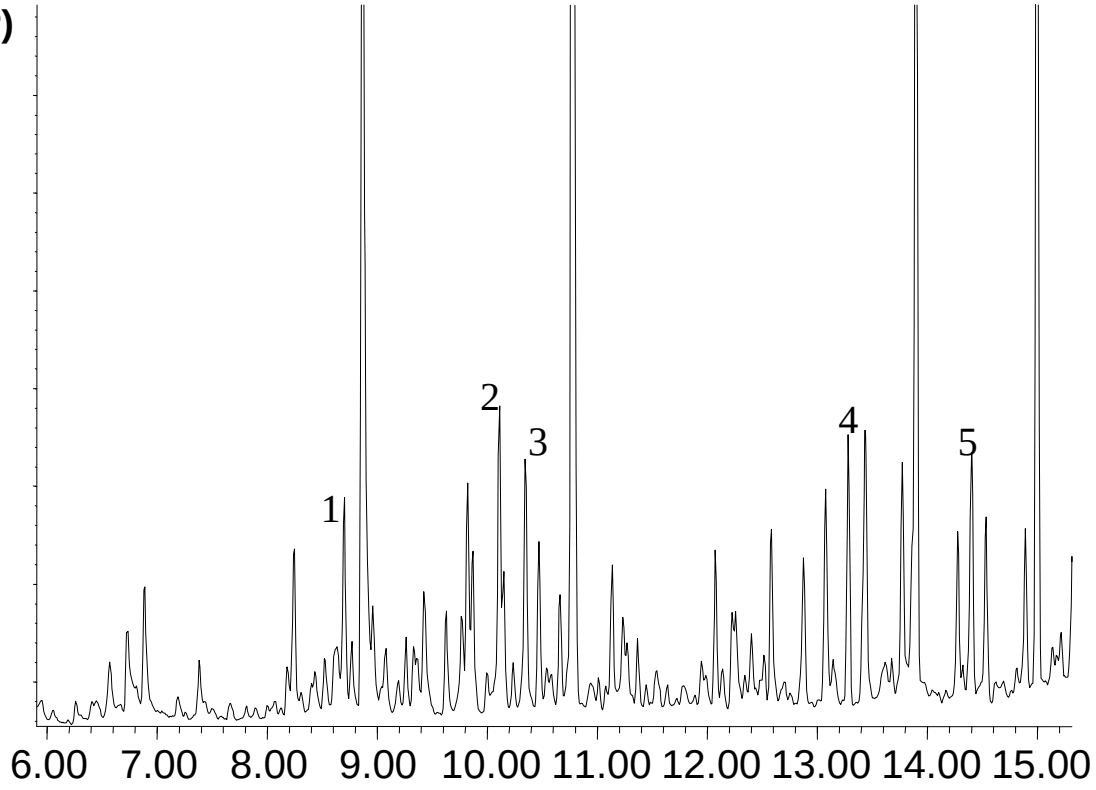


“Dials” to Turn in SPME Methods

Device	Type of coating (polarity) Coating thickness
Sample	Headspace vs. direct immersion extraction Ionic strength, pH, polarity of sample solution Stirring (sample) & agitation (fiber) Extraction time Extraction temperature
Instr.	Inlet liner volume (GC)

SPME Extraction of Odor-Causing Compounds in Water at 2 ppt (GC/MS)

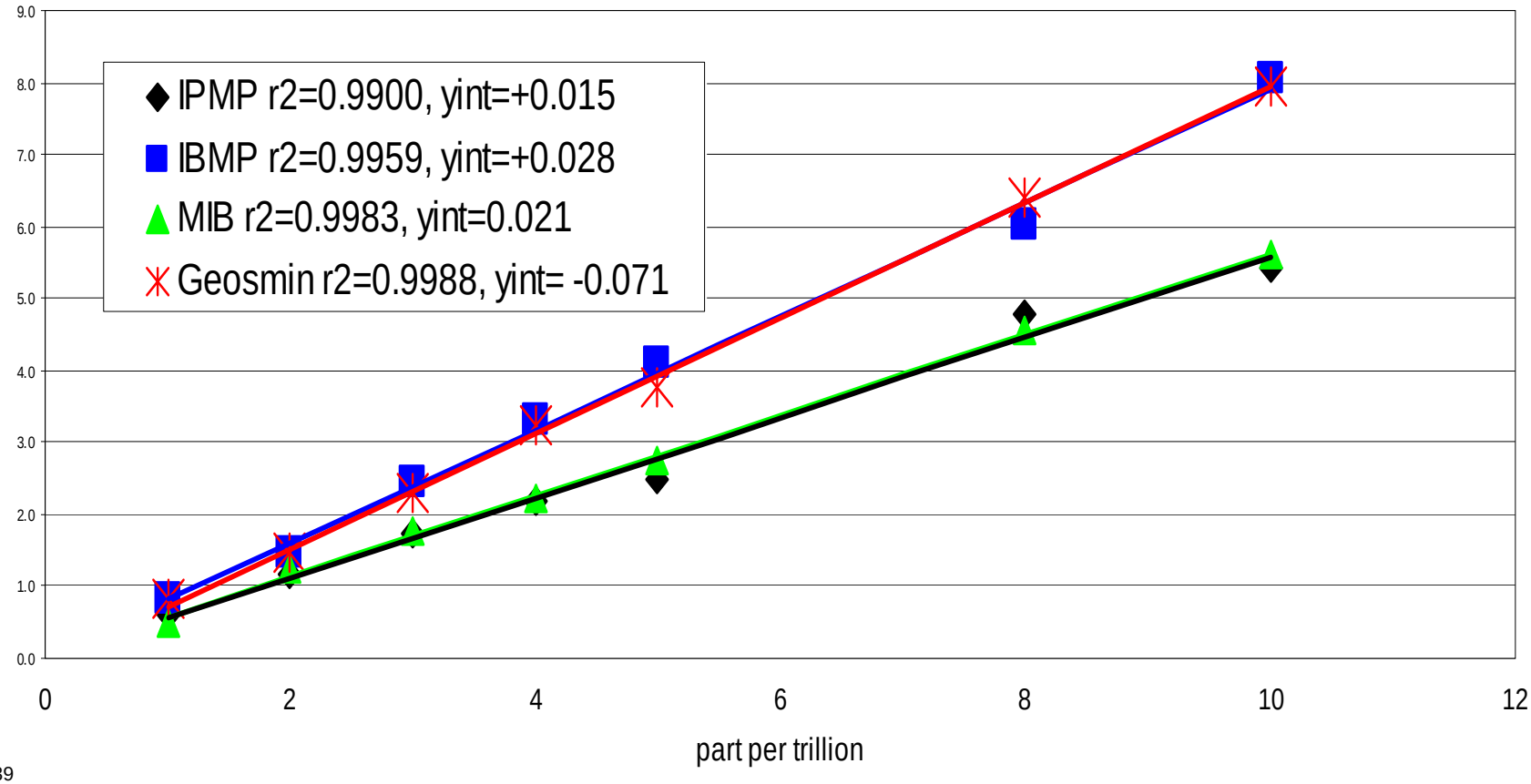
1. **2-Isopropyl-3-methoxypyrazine (IPMP)**
2. **2-Isobutyl-3-methoxypyrazine (IBMP)**
3. **2- Methylisoborneol (MIB)**
4. **2,4,6-Trichloroanisole (I.S. 8ppt)**
5. **(±) Geosmin**



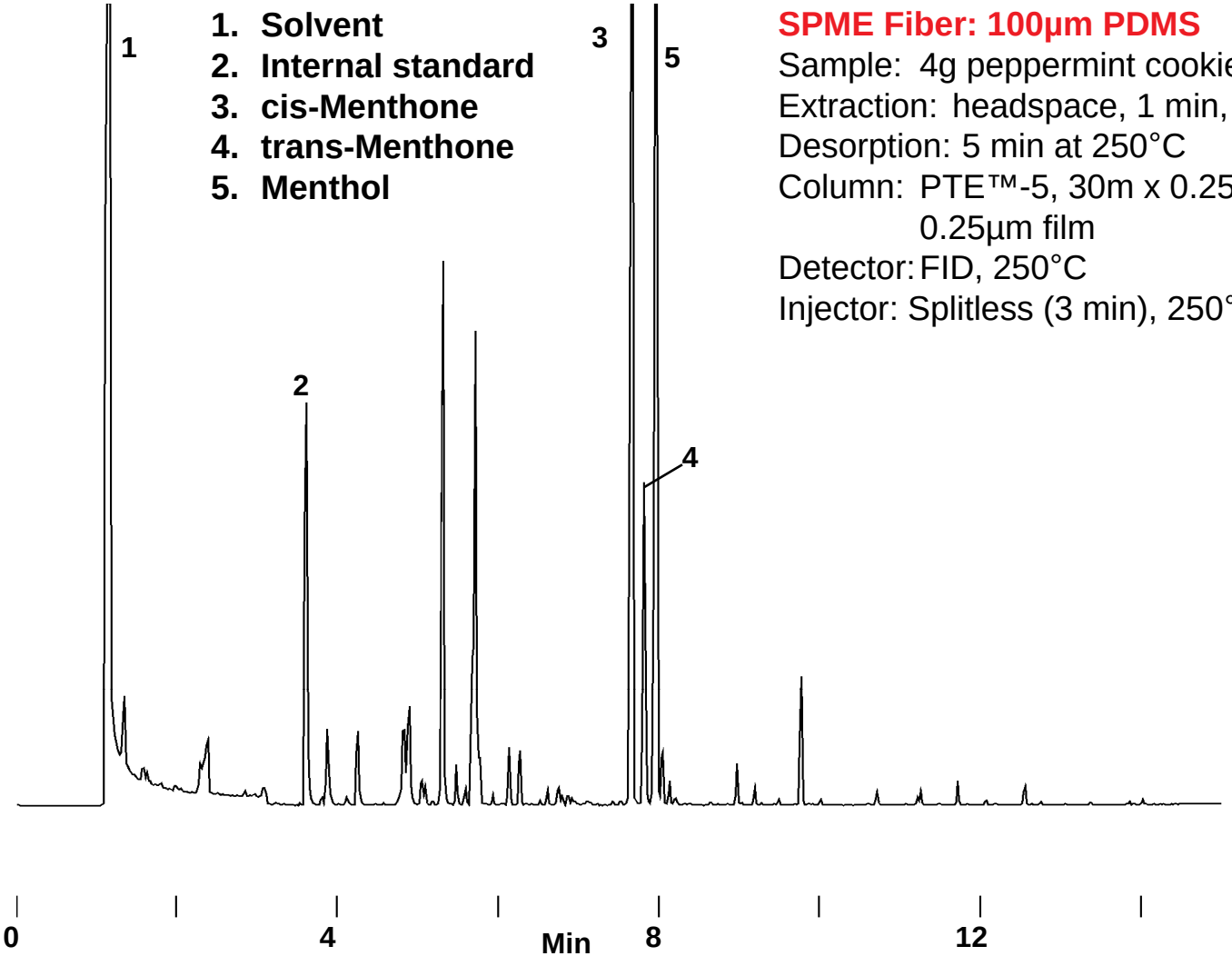
Sample: 25mL water containing odors at 2 ppt 25% NaCl 40mL vial
Fiber: 2cm DVB/Carboxen™/PDMS
Extraction: heated headspace, 30 min, 65°C, rapid stirring
Desorption: 3 min, 250°C, splitter closed
Column: Equity-5, 30m x 0.25mm x 0.25µm film
Oven: 60°C (1 min) to 250°C at 8°C/min
Det.: quadrupole MS, selected ion mode

Quantitative SPME

Linearity of Odor-Causing Compounds from Water at ppt Levels (SPME-GC/MS)



Peppermint Oil in Chocolate Cookie Bar

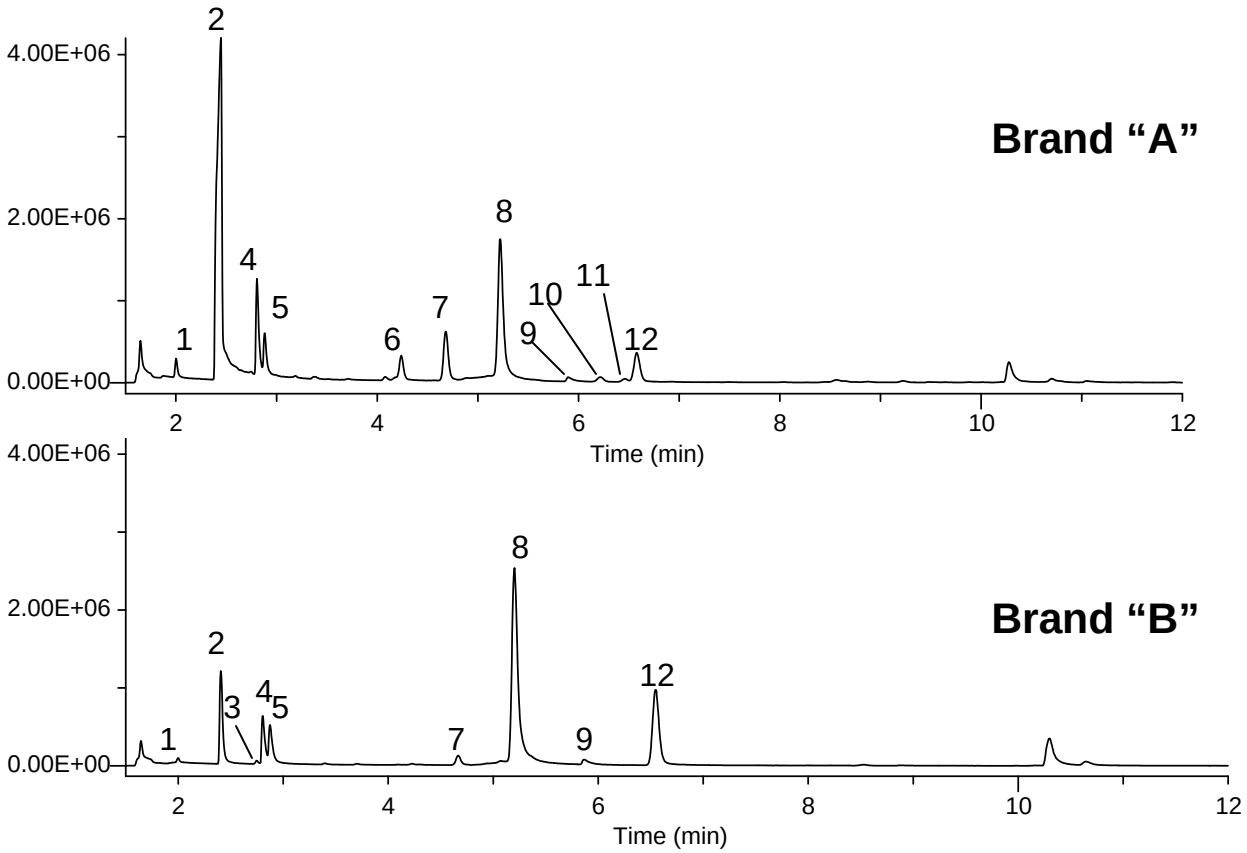


1. **Solvent**
2. **Internal standard**
3. **cis-Menthone**
4. **trans-Menthone**
5. **Menthol**

SPME Fiber: 100µm PDMS
Sample: 4g peppermint cookie bar
Extraction: headspace, 1 min, 45°C
Desorption: 5 min at 250°C
Column: PTE™-5, 30m x 0.25mm ID, 0.25µm film
Detector: FID, 250°C
Injector: Splitless (3 min), 250°C

794-0862

Residual Solvents in Commercial Ibuprofen



1. Acetaldehyde
2. Ethanol
3. Acetonitrile
4. Acetone
5. 2-Propanol
6. 2-Methylpentane
7. 3-Methyl pentane
8. Hexane
9. Ethyl acetate
10. 2,2-Dimethylpentane
11. 2,4-Dimethylpentane
12. Methylcyclopentane

New SPME Research Focus: LC Sample Prep

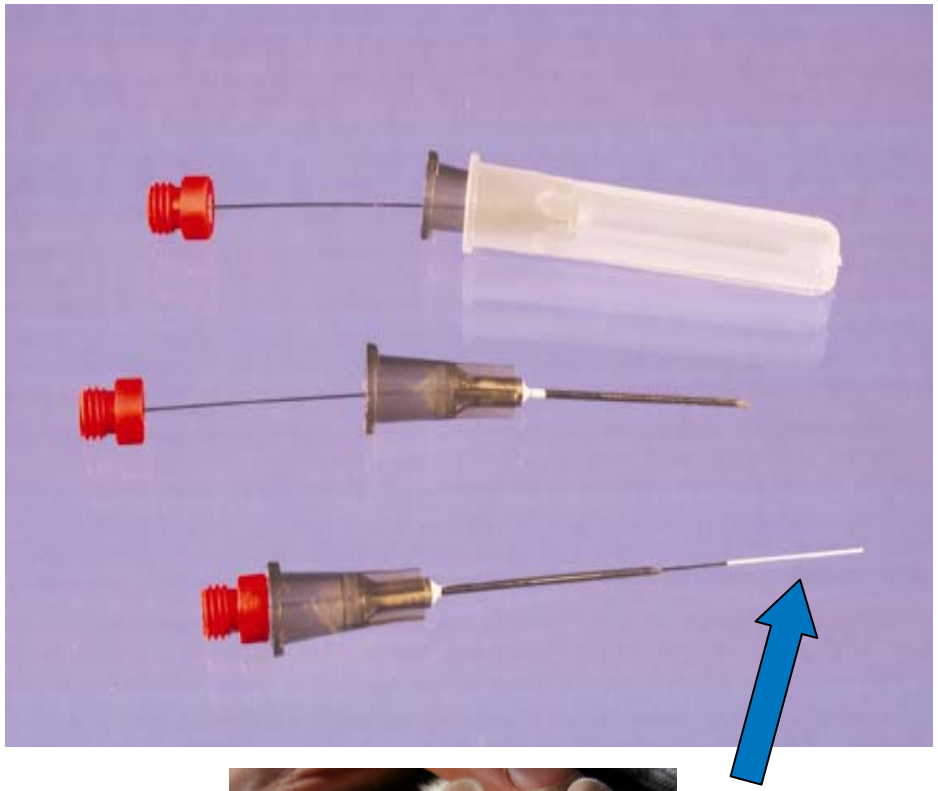
Single use biocompatible fiber probes for *in vivo* analysis

Inert to sample matrix

Comprise C18-silica in a special binder

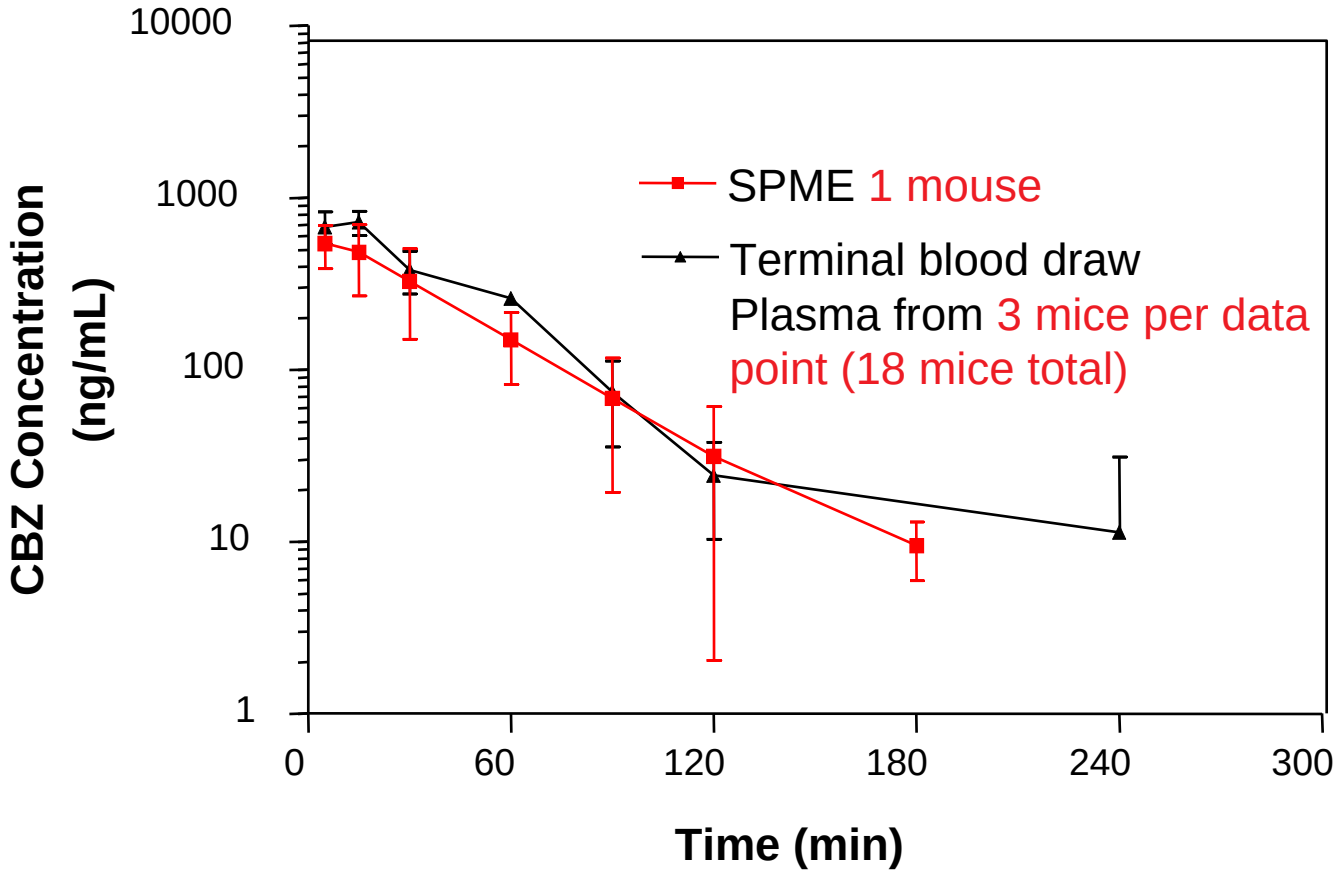
Solvent-stable coatings ideal for:

- Difficult matrixes (plasma, tissue)
- Non-volatile analytes
- Living systems (e.g. animals, plants, cell culture)
- Multiple data points per sample
- Reducing lab animal sacrifice



For laboratory use only

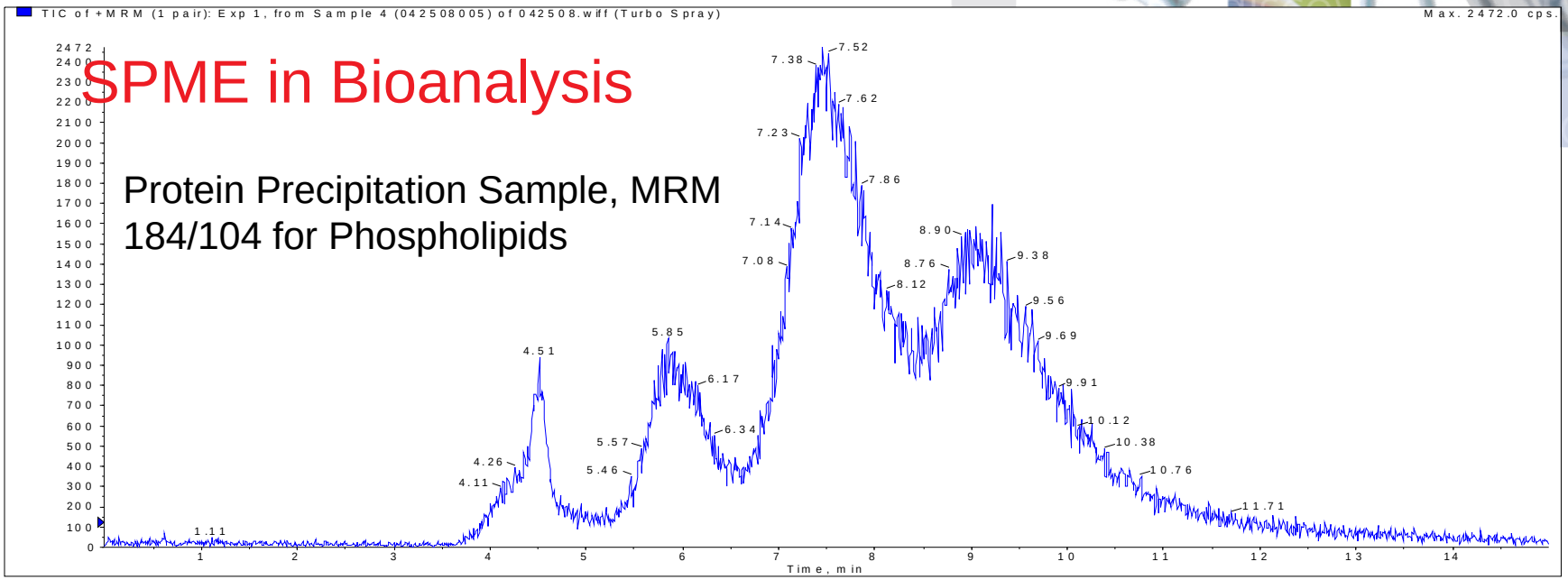
Comparison of SPME *in-vivo* PK Study of Carbamazepine from Mice Whole Blood to Extracts of Plasma Removed from Mice

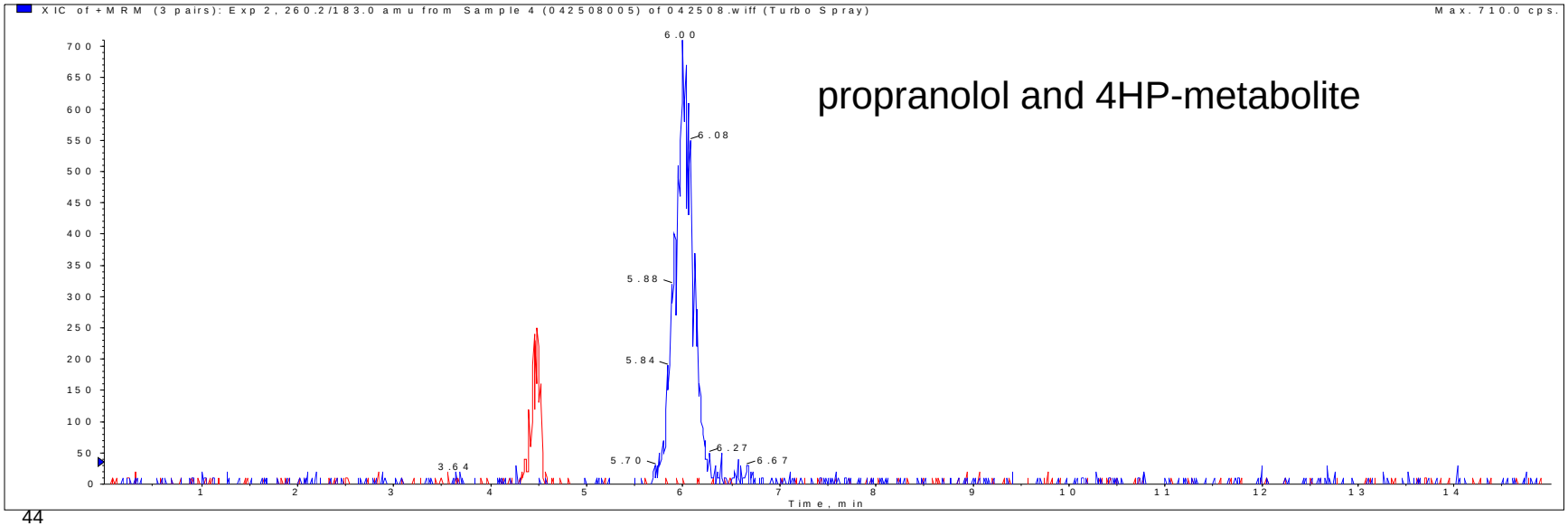


Slide Courtesy of Ines de Lannoy, NoAb BioDiscoveries

SPME in Bioanalysis

Protein Precipitation Sample, MRM 184/104 for Phospholipids

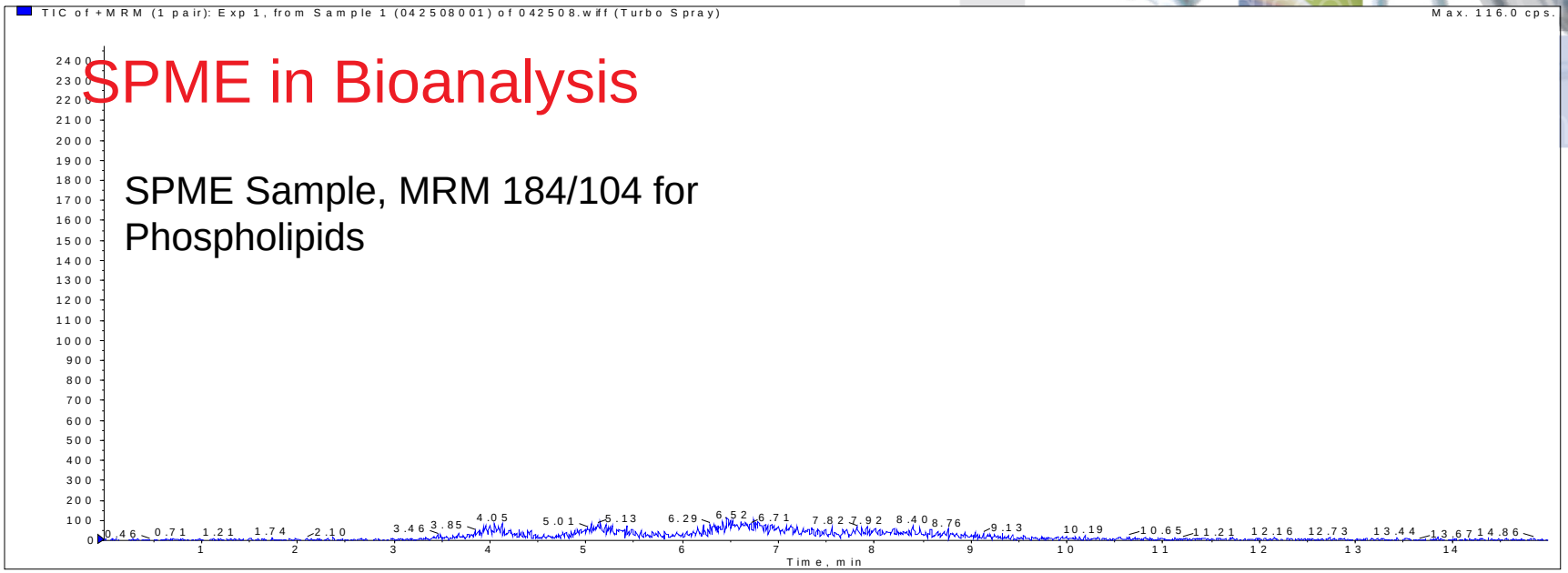


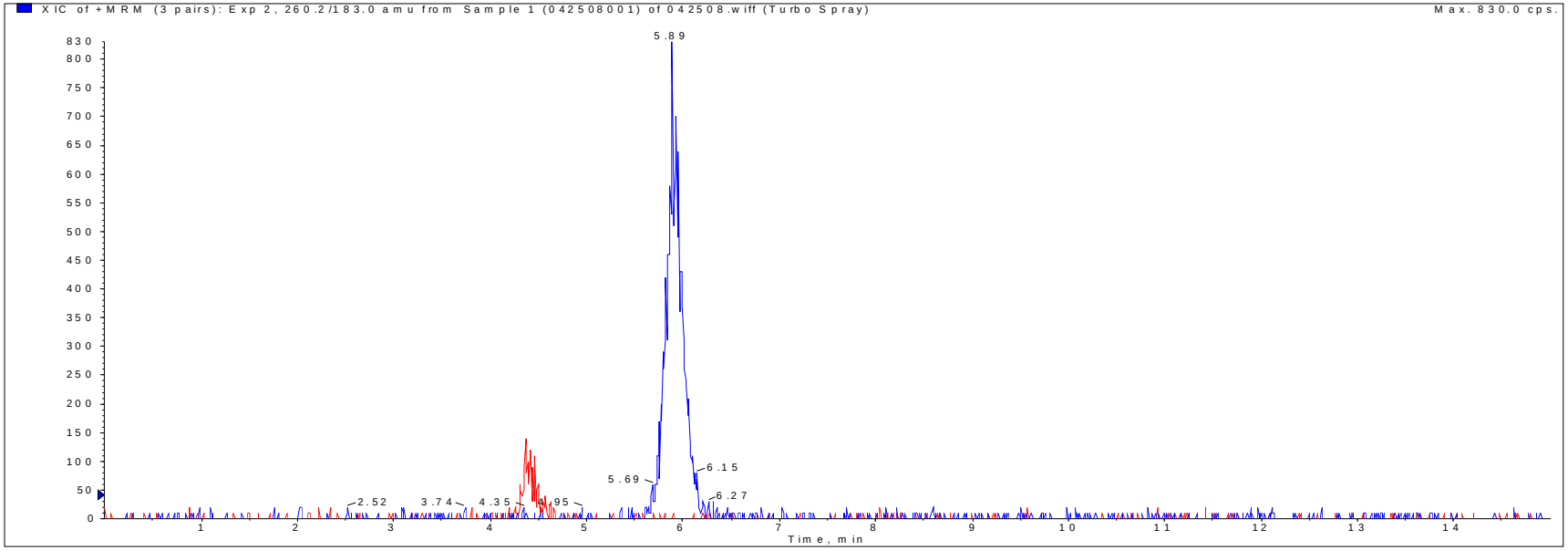


propranolol and 4HP-metabolite

SPME in Bioanalysis

SPME Sample, MRM 184/104 for Phospholipids

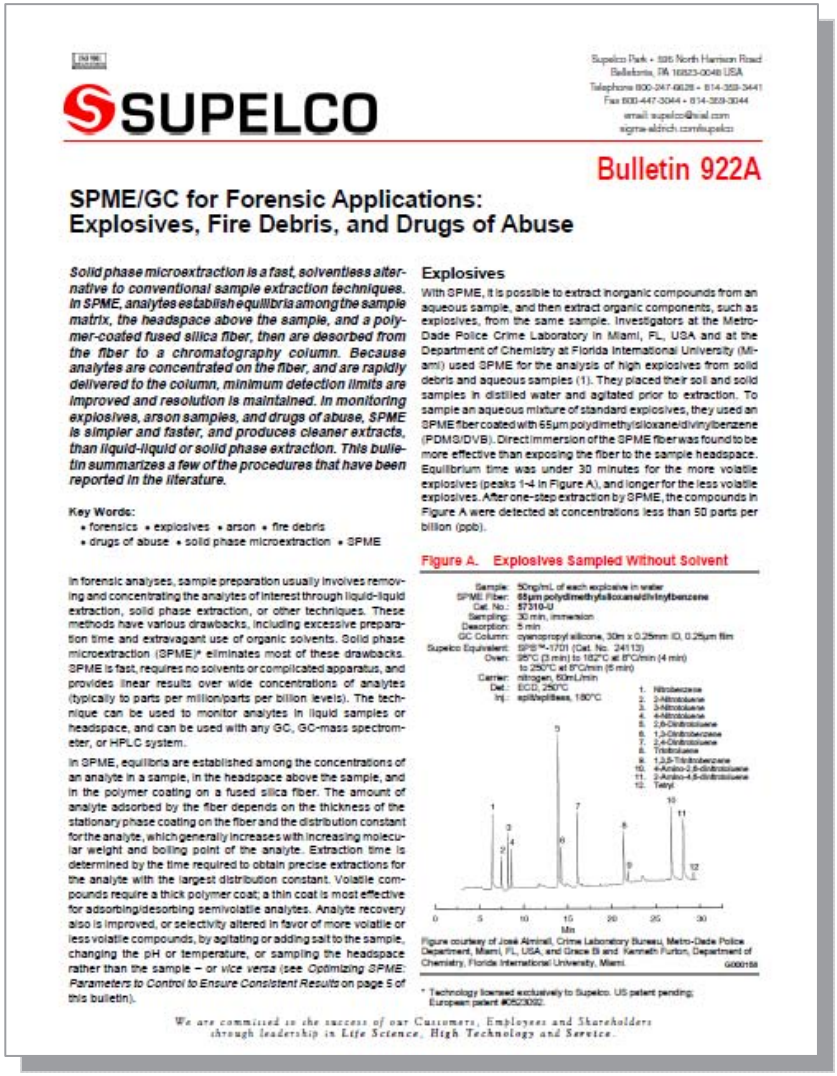




SIGMA-ALDRICH®

SPME Technical Literature

- Forensics
- Food & Beverage
- Flavor & Fragrance
- Environmental
- Biotech
- Pharmaceutical



SUPELCO

Supelco Park • 595 North Harrison Road
Bellefonte, PA 16823-0048 USA
Telephone 800-247-6628 • 814-359-3441
Fax 800-447-3044 • 814-359-3044
email supelco@sial.com
sigma-aldrich.com/supelco

Bulletin 922A

SPME/GC for Forensic Applications: Explosives, Fire Debris, and Drugs of Abuse

Solid phase microextraction is a fast, solventless alternative to conventional sample extraction techniques. In SPME, analytes establish equilibria among the sample matrix, the headspace above the sample, and a polymer-coated fused silica fiber, then are desorbed from the fiber to a chromatography column. Because analytes are concentrated on the fiber, and are rapidly delivered to the column, minimum detection limits are improved and resolution is maintained. In monitoring explosives, arson samples, and drugs of abuse, SPME is simpler and faster, and produces cleaner extracts, than liquid-liquid or solid phase extraction. This bulletin summarizes a few of the procedures that have been reported in the literature.

Key Words:
• forensics • explosives • arson • fire debris
• drugs of abuse • solid phase microextraction • SPME

In forensic analyses, sample preparation usually involves removing and concentrating the analytes of interest through liquid-liquid extraction, solid phase extraction, or other techniques. These methods have various drawbacks, including excessive preparation time and extravagant use of organic solvents. Solid phase microextraction (SPME)* eliminates most of these drawbacks. SPME is fast, requires no solvents or complicated apparatus, and provides linear results over wide concentrations of analytes (typically to parts per million/parts per billion levels). The technique can be used to monitor analytes in liquid samples or headspace, and can be used with any GC, GC-mass spectrometer, or HPLC system.

In SPME, equilibria are established among the concentrations of an analyte in a sample, in the headspace above the sample, and in the polymer coating on a fused silica fiber. The amount of analyte adsorbed by the fiber depends on the thickness of the stationary phase coating on the fiber and the distribution constant for the analyte, which generally increases with increasing molecular weight and boiling point of the analyte. Extraction time is determined by the time required to obtain precise extractions for the analyte with the largest distribution constant. Volatile compounds require a thick polymer coat; a thin coat is most effective for adsorbing/desorbing semivolatile analytes. Analyte recovery also is improved, or selectivity altered in favor of more volatile or less volatile compounds, by agitating or adding salt to the sample, changing the pH or temperature, or sampling the headspace rather than the sample – or *vice versa* (see *Optimizing SPME: Parameters to Control to Ensure Consistent Results* on page 5 of this bulletin).

Explosives

With SPME, it is possible to extract inorganic compounds from an aqueous sample, and then extract organic components, such as explosives, from the same sample. Investigators at the Metro-Dade Police Crime Laboratory in Miami, FL, USA and at the Department of Chemistry at Florida International University (Miami) used SPME for the analysis of high explosives from solid debris and aqueous samples (1). They placed their soil and solid samples in distilled water and agitated prior to extraction. To sample an aqueous mixture of standard explosives, they used an SPME fiber coated with 65µm polydimethylsiloxane/divinylbenzene (PDMS/DVB). Direct immersion of the SPME fiber was found to be more effective than exposing the fiber to the sample headspace. Equilibrium time was under 30 minutes for the more volatile explosives (peaks 1-4 in Figure A), and longer for the less volatile explosives. After one-step extraction by SPME, the compounds in Figure A were detected at concentrations less than 50 parts per billion (ppb).

Figure A. Explosives Sampled Without Solvent

Sample: 50ng/mL of each explosive in water
SPME Fiber: 65µm polydimethylsiloxane/divinylbenzene
Cat. No.: 57310-U
Sampling: 30 min, immersion
Desorption: 5 min
GC Column: cyanopropyl silicone, 30m x 0.25mm ID, 0.25µm film
Supelco Equivalent: SPB™-1701 (Cat. No. 24113)
Oven: 95°C (3 min) to 162°C at 8°C/min (4 min) to 250°C at 8°C/min (5 min)
Carrier: nitrogen, 50mL/min
Det.: ECD, 250°C
Inj.: split/splitless, 180°C

1. Nitrobenzene
2. 2-Nitrotoluene
3. 3-Nitrotoluene
4. 4-Nitrotoluene
5. 2,6-Dinitrotoluene
6. 1,3-Dinitrobenzene
7. 2,4-Dinitrotoluene
8. Trinitrotoluene
9. 1,3,5-Trinitrobenzene
10. 4-Amino-2,6-dinitrotoluene
11. 2-Amino-4,6-dinitrotoluene
12. Tetryl

Figure courtesy of José Almirall, Crime Laboratory Bureau, Metro-Dade Police Department, Miami, FL, USA, and Grace Bi and Kenneth Furton, Department of Chemistry, Florida International University, Miami. G000158

* Technology licensed exclusively to Supelco. US patent pending; European patent #0523092.

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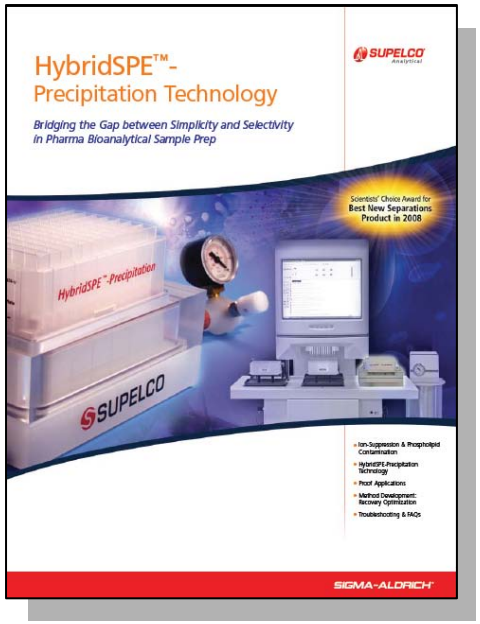
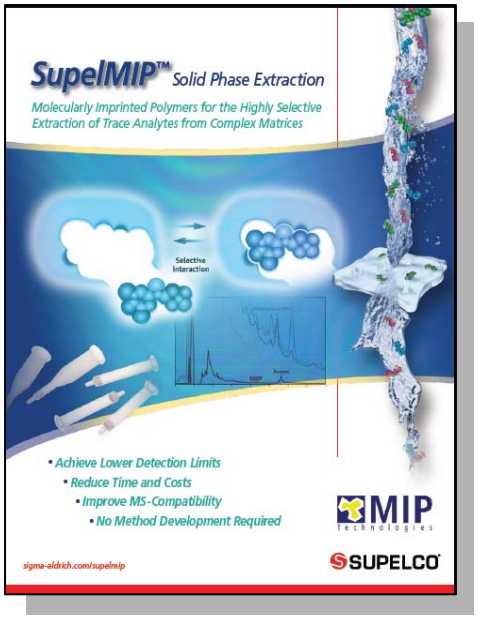
SIGMA-ALDRICH®

Sample Prep Innovations

- HybridSPE™-PPT for reducing matrix effects
- SupelMIP™ high-specificity sample prep devices
- SPME, including biocompatible fibers





Other Supelco Sample Prep Devices

General Purpose

- Polymer SPE
- Flash chromatography
- Carbonaceous adsorbents

Food/Food Safety

- Dispersive SPE (QuEChERS) (pesticides)
- DSC-SCX (melamine)
- Silver ion SPE (FAMEs)
- Dual-layer SPE (pesticides)

Biochromatography

- Packed pipette tips

Synthetic reaction mixtures

- Mixed-mode SPE (DSC-MCAX)

Environmental

- Radiello® Passive-Diffusive air sampling products
- BPE/DNPH for simultaneous measurement of ozone and carbonyls
- Supelclean Sulfoxide (PCBs in oil)
- Supelpak 2 (purified XAD-2)
- Dioxin Prep System
- Mercury sampling tubes
- Deactivated Thermal Desorption Tubes

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Prof. Daniel Armstrong, U. Texas Arlington (SPME photomicrographs, chiral, ionic liquids, …)

Supelco and Fluka R&D Teams

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