Automated Fractionation System that Increases Recovery and Optimize Precision of Solid Phase Extraction Methodology

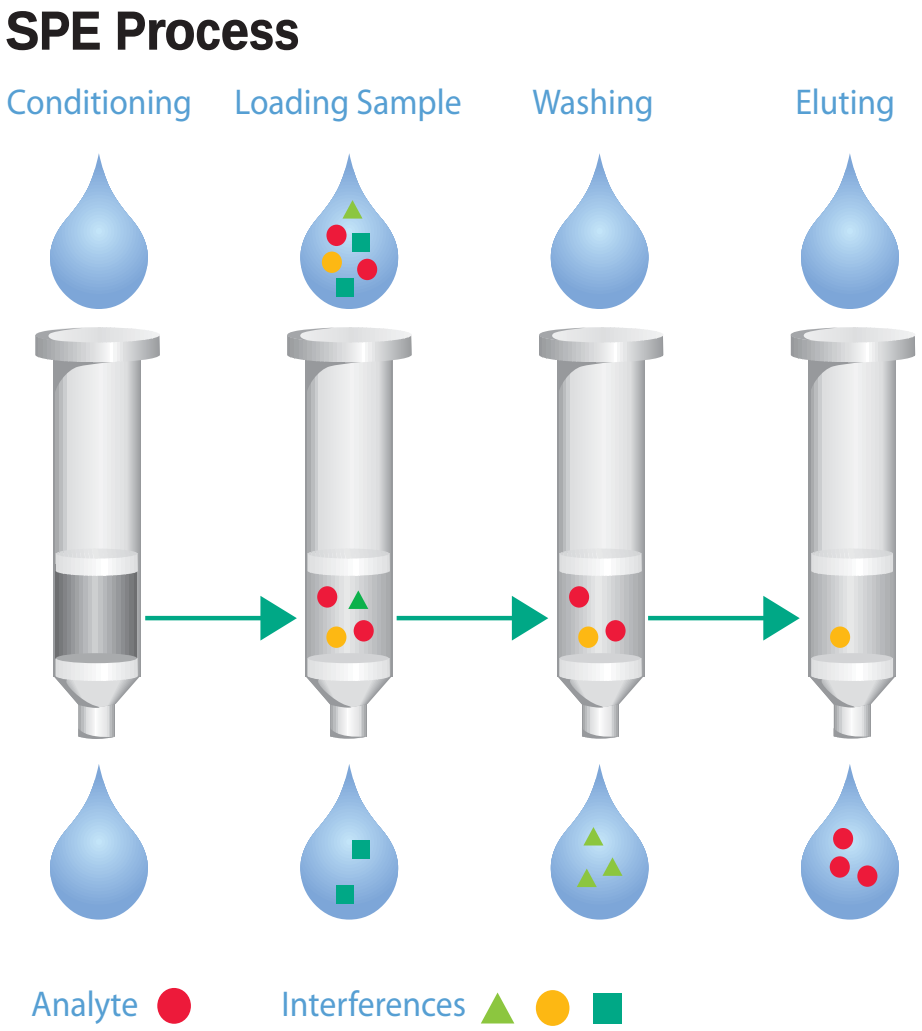
Joan M. Stevens, Ph.D., and Mark Crawford
Gilson, Inc., 3000 Parmenter Street., Middleton, WI 53562
www.gilson.com

Introduction:

- Solid Phase Extraction has become a technique of choice for sample cleanup and trace enrichment
- Some of the benefits of SPE: Versatility, Selectivity, Speed, and Low Solvent usage
- There are other methods for sample cleanup: Liquid-Liquid Extraction, Protein Precipitation, Turbulent Flow, and Dialysis
- Most automated methods find their roots in a manual method
- Most manual methods don't optimize the extraction mechanism due to the so called "time" believed to be involved in method development
- Automation in its most general term removes the repetitive/monotonous nature associated with method development and the drain on human capital

Optimization

- Condition
  - Solvent
  - Volume
- Load
  - Volume
  - Break-through
- Wash
  - Volume
  - Wash solvent selection
  - Break-through
- Elute
  - Volume
  - Elution solvent selection
  - Percent recovery



Let's Face Facts:

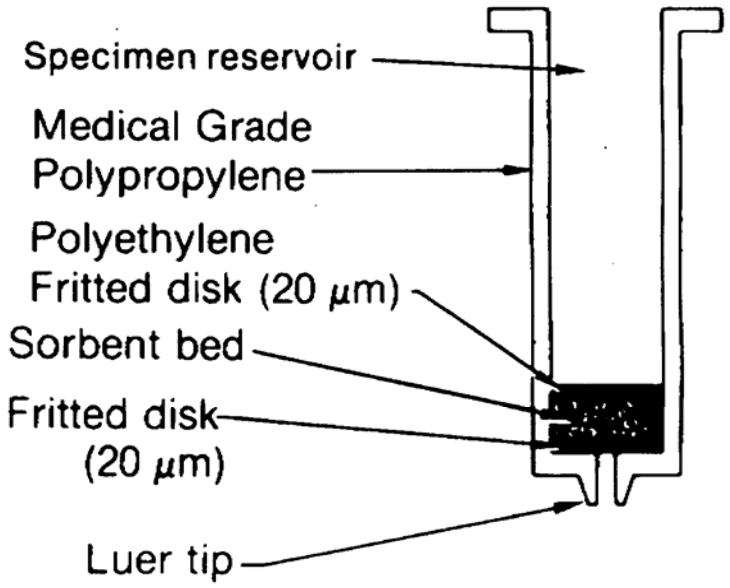
- Time is a limiting factor
- Although we would appreciate all the steps associated with SPE to be optimized work load doesn't allow us this luxury
- However if an automated system could accomplish all the variations of solutions, additions and elutions then we could optimize our SPE methods without manual intervention and still reap the benefits

Just the Basic Facts:

- **A typical SPE method with disposable cartridges or 96 well plates consists of 4 basic steps:**

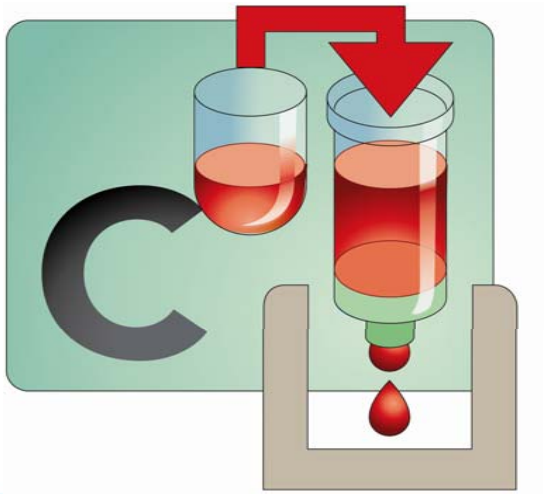
CLWE

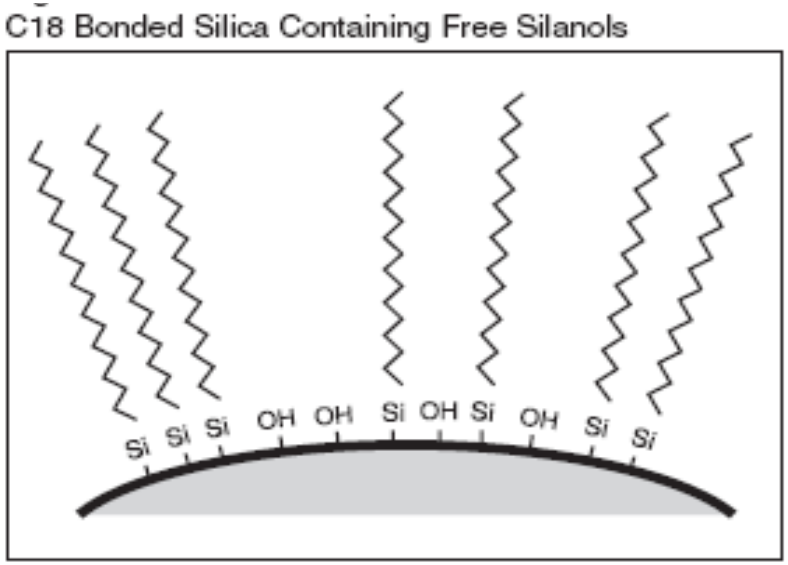
- **C = Condition**
  - **Let's think about this**
  - **What is a SPE cartridge?**
  - **Generally speaking its: A dry sorbent/material held in place with a frit on the bottom and one on the top**



Conditioning:

- Wets/Activates the packing bed by solvation
- Very important step often ignored or minimized
- Major step DO NOT LET THE CARTRIDGE DRY OUT
- Methanol is usually the initial conditioning step
  - Hydrates the particles, remember the packing material isn't as efficient as the particles found in an HPLC column
  - Need to rely on the basics of science to illicit the required effect





Now What?

- **Sample what sample:**
  - **What do you know about the sample**
    - **Serum**
    - **Plasma**
    - **Urine**

- **Why is Serum and Plasma difficult to work with?**

  •Because of protein binding

  •A particular drug can be bound to proteins in the biological fluid that will inhibit its interaction with the SPE cartridge, usually greater than 50% bound

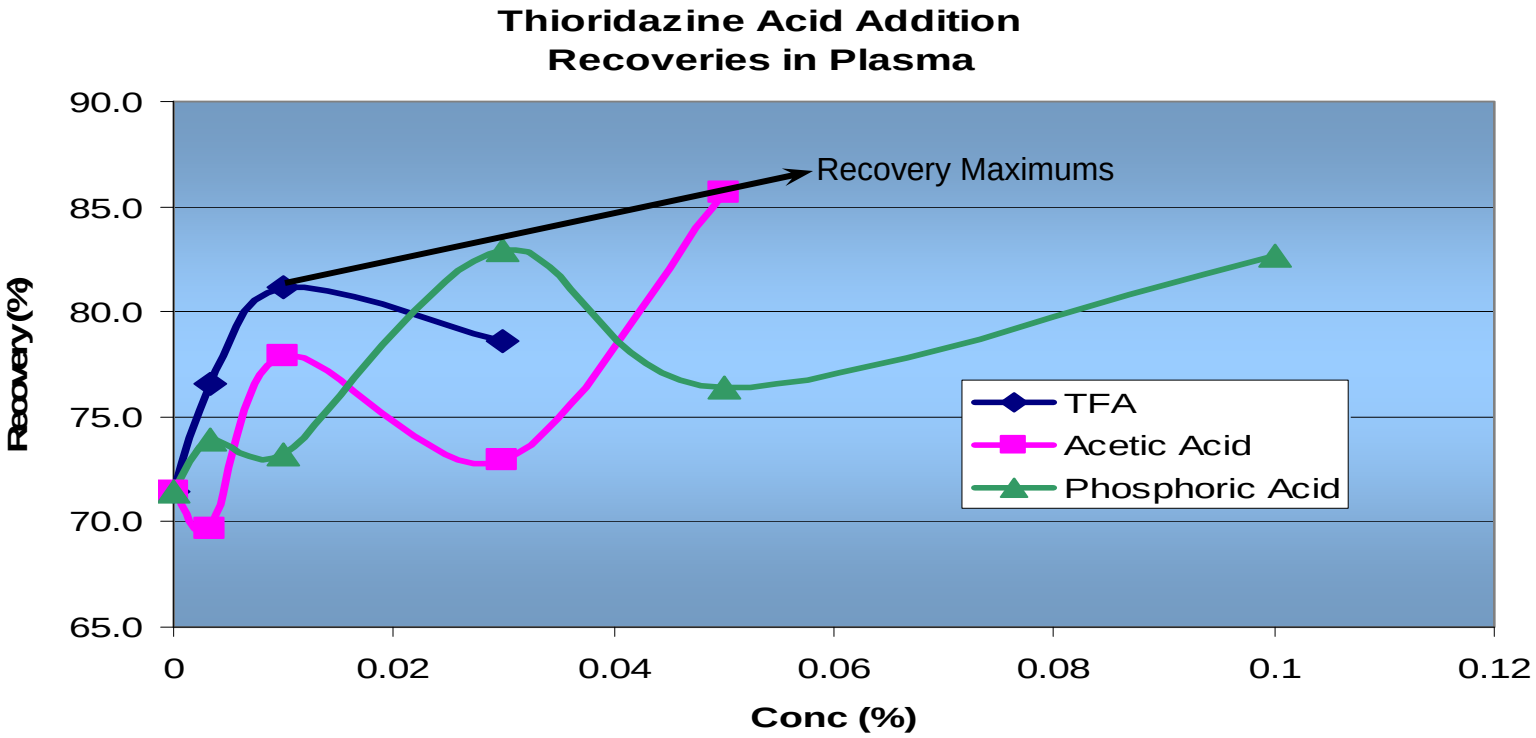
Interrupting Protein Binding:

- **Prior to loading the sample onto the SPE cartridge protein interactions with the drug need to be interrupted, if not the results will be compromised**
- **This is accomplished with the addition of acidic solution or basic solution**
- **Optimization of common acidic additions is shown below:**

	Acid	Chlorpromazine		Thioridazine	
Acid	Conc. (%)	Rec. (%)	CV (%)	Rec. (%)	CV (%)
None	0	88	3.0	71	4.0
TFA	0.0033	96	2.0	77	2.3
TFA	0.01	96	1.8	81	2.3
TFA	0.03	92	1.1	79	1.7
Acetic	0.0033	88	0.9	70	1.4
Acetic	0.01	94	2.8	78	3.2
Acetic	0.03	91	2.4	73	2.8
Acetic	0.05	95	1.7	86	1.6
H_3PO_4	0.0033	89	1.6	74	3.2
H_3PO_4	0.01	89	1.9	73	1.6
H_3PO_4	0.03	98	1.7	83	1.8
H_3PO_4	0.05	90	2.3	76	1.8
H_3PO_4	0.1	97	1.7	83	1.8

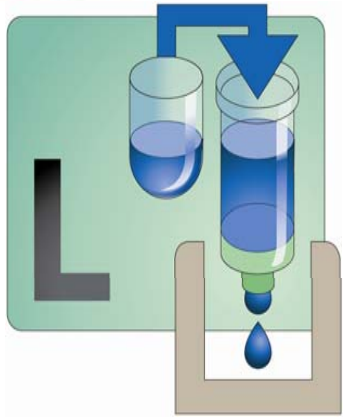
Optimum

Plasma Acid Addition Recoveries:

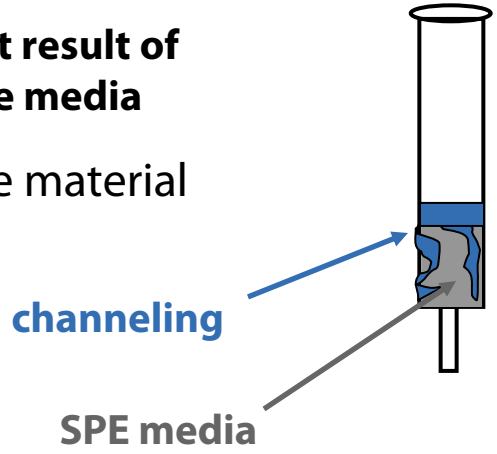


Graph 1: As the acids get weaker, the recovery maximums increase, indicating that a weaker acid is more efficient to control plasma matrix effects.

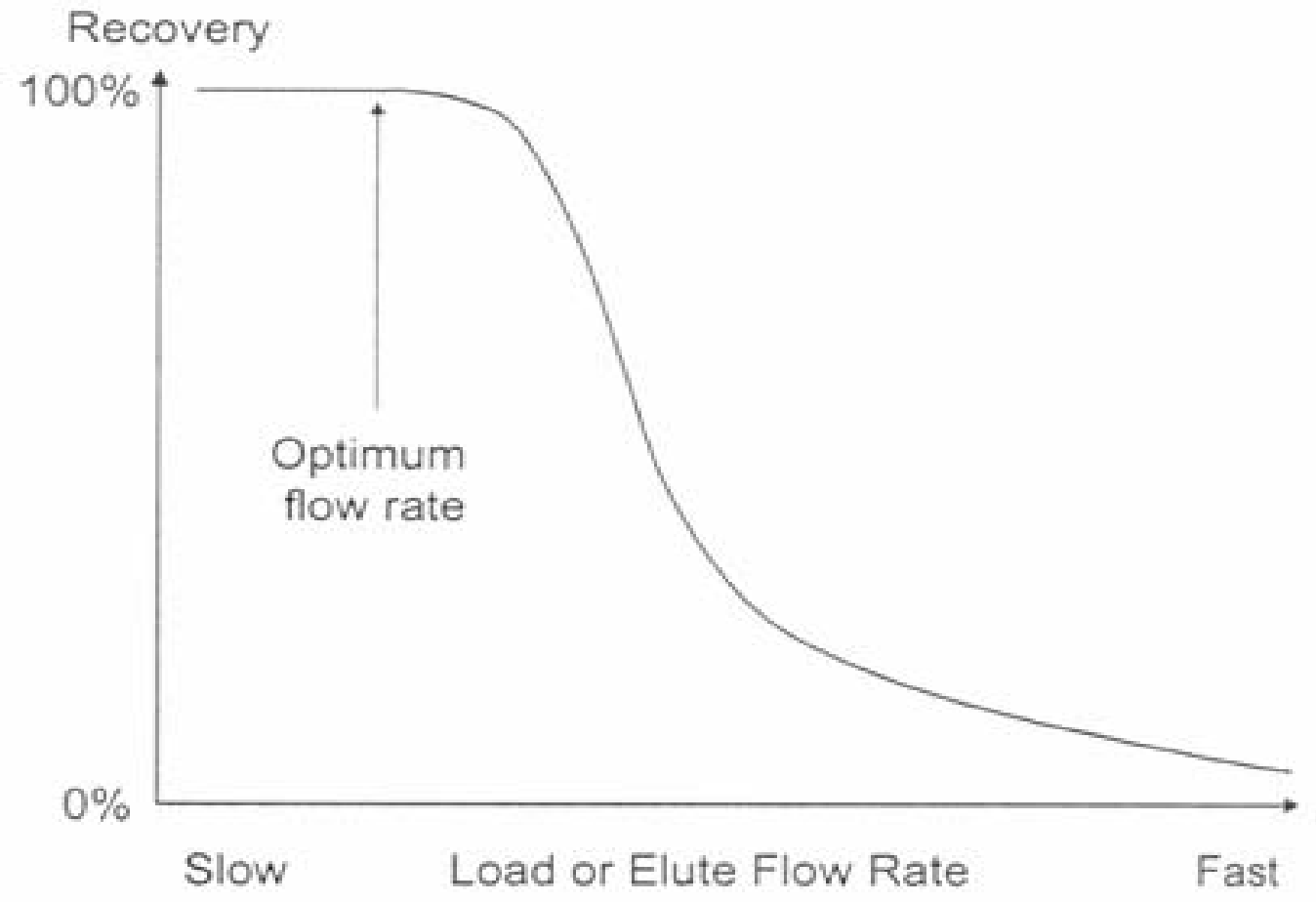
Loading the Sample:



- **Load the sample in two steps is possible why?**
  - **Take a look at the process**
    - **The media is activated----→ expose the media to the sample**
    - **The longer the sample can interact with the media pushes the attraction of sample with media in the forward direction**
    - **If the sample can be added in two shots the first interaction with the sample allows the media to relax and therefore be more accepting of the remaining sample**
    - **Under the same concept the sample must be added slowly to the SPE cartridge usually 1 ml/min ~20 drops /minute**
    - **Channeling is an issue in SPE cartridges a direct result of introducing the sample much too quickly to the media**
    - The sample must become *"intimate"* with the material



Relationship Between Sample Recovery and Flow Rate in SPE

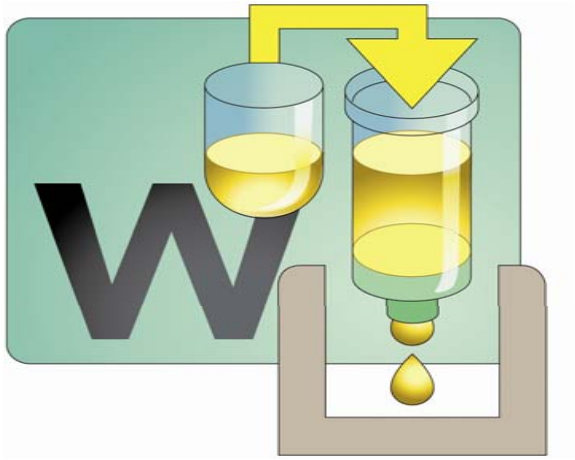


How Fragile is Fragile:

- **Now what do I do?**
  - **The sample in now associated with the cartridge material….**
  - **The sample isn't going anywhere at this point a nice not too aggressive push of positive pressure or vacuum will pull the liquid through the cartridge**
  - **You will NOT dry the cartridge out, how long does it take to remove a aqueous solution in an evaporator?**
  - **It will require several minutes of constant gas flow or vacuum to dry the cartridge out**

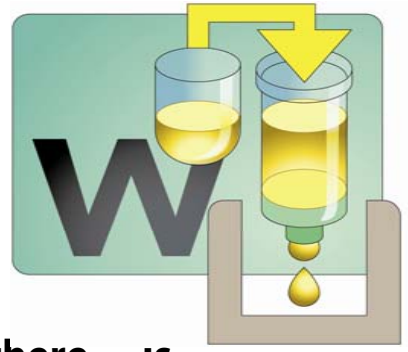
Washing the Cartridge:

- ***"Throwing the baby out with the bath water"***
- Optimization of this step is crucial



Washing- Before Elution

- These two steps are not to associated with each other however they are…
- What are my concerns:
  - Adequate Recovery: Will my recovery be adequate?????
  - Reproducibility: Between labs within my lab
    - Confidence Level: Does the assay transfer….how?



- We need to test what is being eluted

  •As much as inferents always compromise the purity of the effluent there is a fine line between washing the cartridge enough to remove interferents without also decreasing the recovery of the analyte of interest

  •How do we do it …..possibly automate the array to find the optimum set of conditions

  • Rule of thumb if it elutes with 40% wash use 5/10% of two washes slowly

  • Test the wash solutions for breakthrough of the analyte if mass spec is available choose it as your detection mode

Compound – Sorbant Interaction

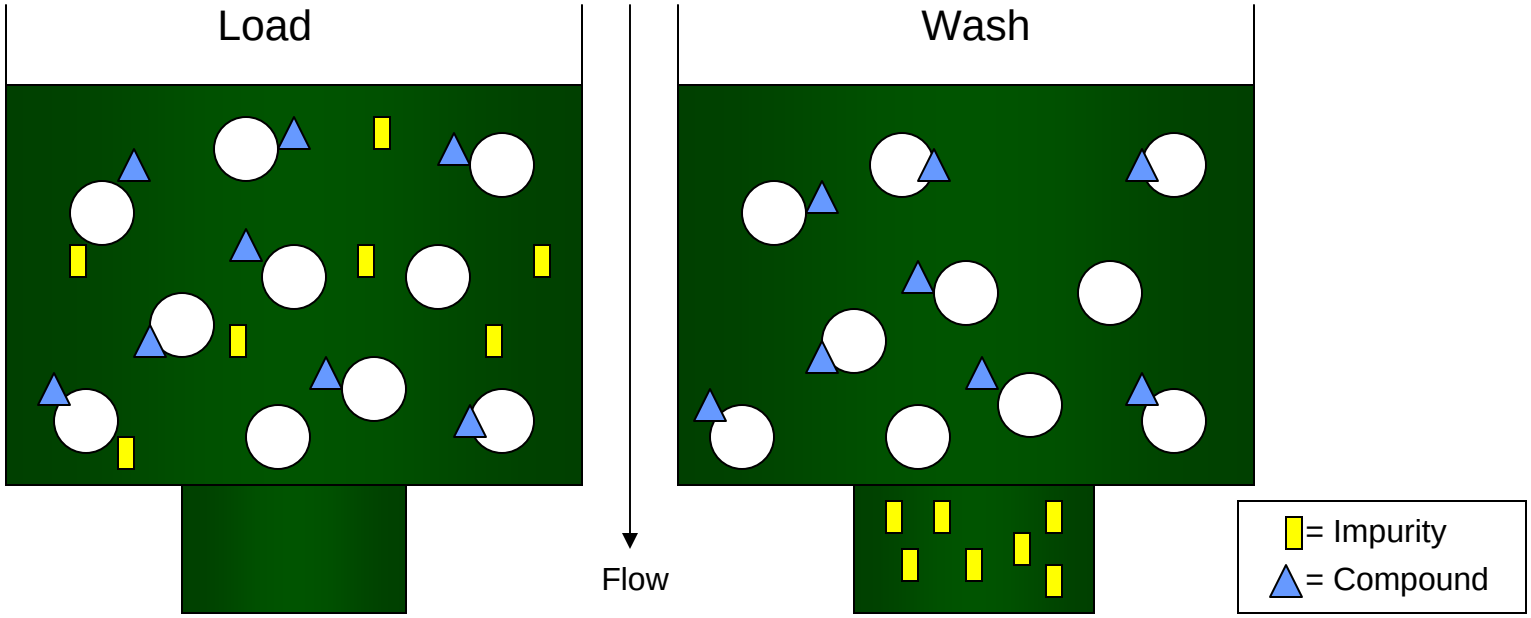


Diagram 1: The compound-sorbant interaction is what makes solid phase extraction work. The solid phase interacts with the sample based on chemical structure (polarity, functionalities, size). Reverse phase methodology allows the sample to grip to the sorbant due to hydrophobicity. The compounds therefore is attracted to the sorbant until washed away by an organic solvent. If the solvent is strong enough the sorbant lets go of the compound and elutes. The trick is to be able to wash the impurities that are soluble in water and low organic solvents without eluting the compounds of interest.

Optimizing the Wash and Elution Step: Automated

- There are numerous SPE methods available, the web is a wonderful tool for perusing SPE manufactures sites for methods; textbooks, published references, applications bibliographies

- Choose a method that extracts a drug/compound with some or all of the same functionality associated with your compound
  - Amines, Carboxylic acids, Hydrophobicity

- Although the method may not be exact this is a wonderful starting point

- Automating the method for optimization is now accessible

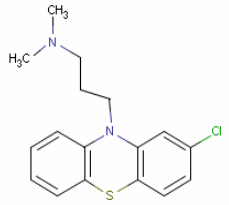
Automated Optimization:

- Simple Liquid Handler and SPE racks
- The SPE rack allow for multiple elutions or fractionation with various solvents to optimize each and every step of an SPE method without manual intervention

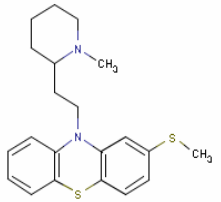
Example Method:

- Human plasma containing Chlorpromazine and Thioridazine at 0.5 mg/ml concentration was acidified with acetic acid 0.05% to interfere with protein binding
- Basic procedure:
  - Condition:1 mL MeOH, 1 mL Water
  - Load: 1 mL of sample (0.5 mLs x 2)
  - Wash: 1 mL Water (x2), 0.5 mL 25% ACN/Water
  - Elute: 0.5 mL (x2) Elution solution (2:2:1, MeOH:ACN:Buffer)
    - Buffer: 4.5 mL Phosphoric Acid, 4.5 mL TEA in 1 liter of water

Chlorpromazine [USAN:BAN:INN]
RN: 50-53-3



Thioridazine [USAN:BAN:INN]
RN: 50-52-2



- SPE method was downloaded from a web site
- The procedure is an example, the method is given as a starting point, they are designed to offer all the basic data that you'll need to make the procedure

Optimization of Method:

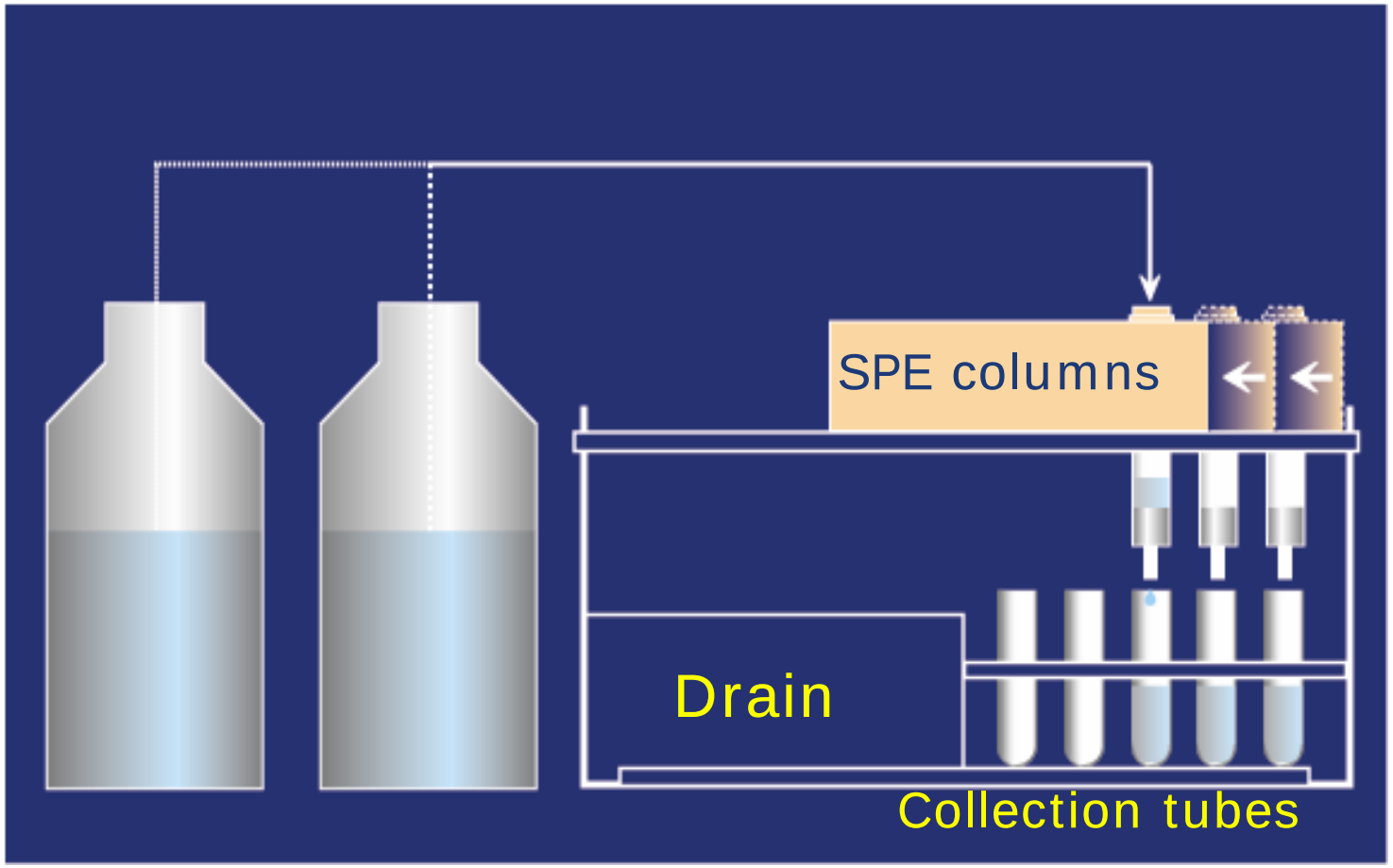
- As mentioned previously the conditioning steps prior to loading the sample is not as important (excluding drying the cartridge) as how the sample is loaded and the wash solutions/elution solutions used relative to recovery
  - To prove the optimization concept a series of various wash solutions and elution solutions were used in the SPE procedure and all wash/elution solutions were collected and analyzed for breakthrough of the compounds in the wash solutions and recovery in the eluent
  - Wash Solution Used
    - **Literature: 25% ACN/Water**
    - Solutions: 10% ACN/Water
      15% ACN/Water
      20% ACN/Water
      25% ACN/Water
      30% ACN/Water

- Elution Solution Used
  - **Literature: 2:2:1 MeOH:ACN:Buffer**
  - Solutions: 1:3:1 MeOH:ACN:Buffer
    3:1:1 MeOH:ACN:Buffer
    4:1 ACN:Buffer

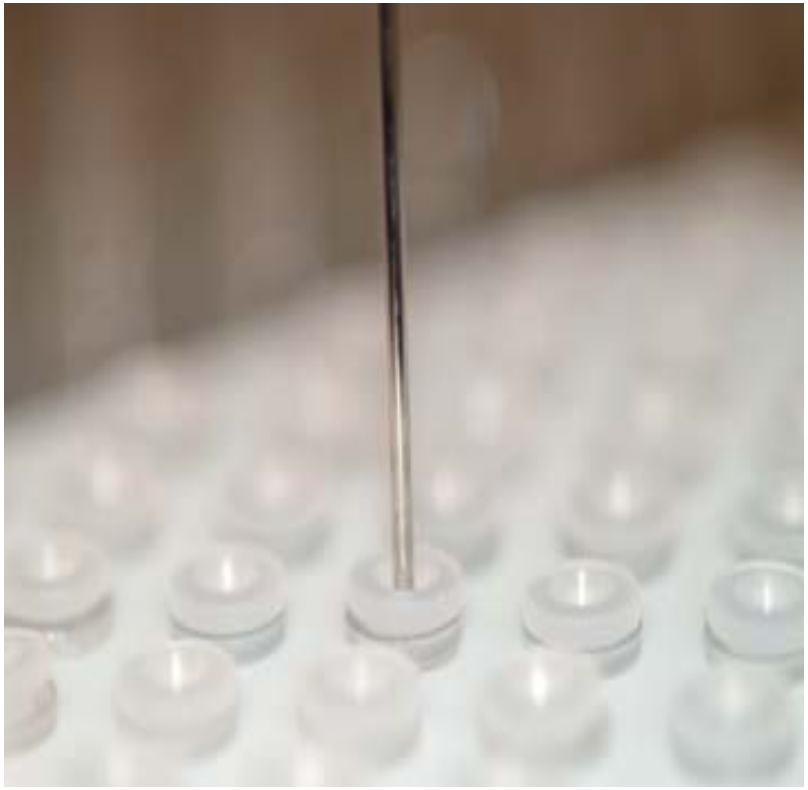
Optimization Grid:

- 5 wash solutions and 4 elution solutions
- Using the basic elution solution 2:2:1 the SPE cartridges were exposed with the various wash solutions repeated 8 times
- Once the optimum wash solution was determined then the elution solution was varied repeated 8 times
- Even with this relatively small study close to 100 samples will be generated

Multi-Collect to Optimize SPE Procedure:

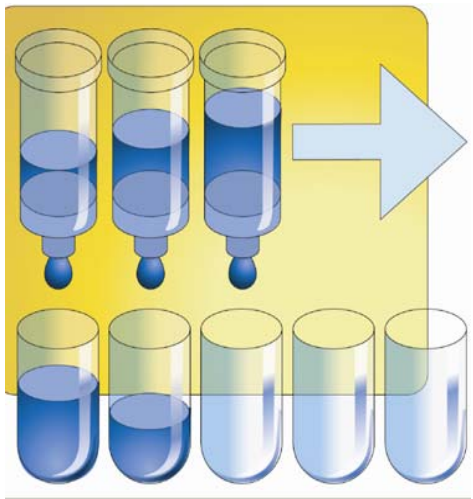


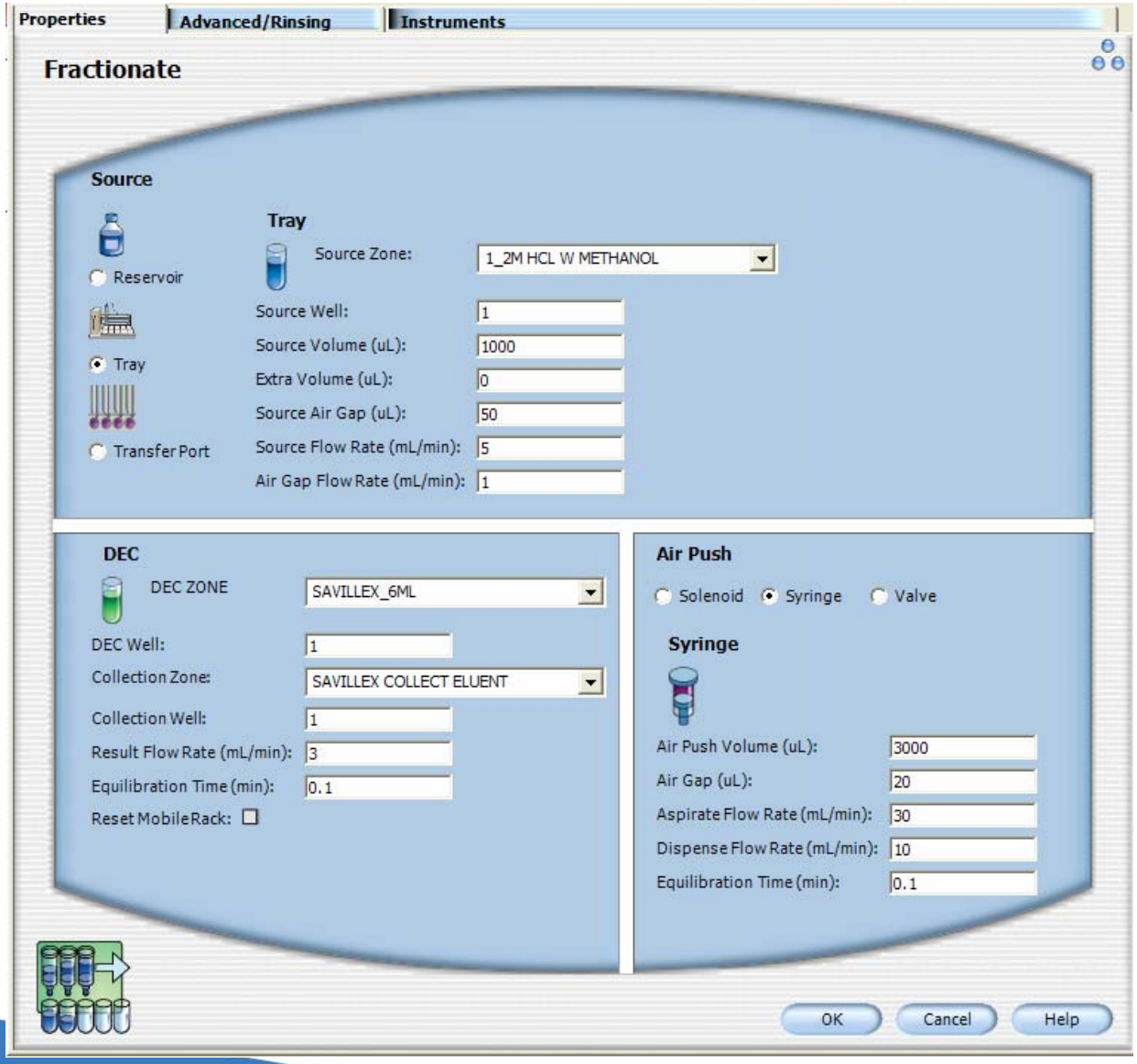
Automated Optimization System:



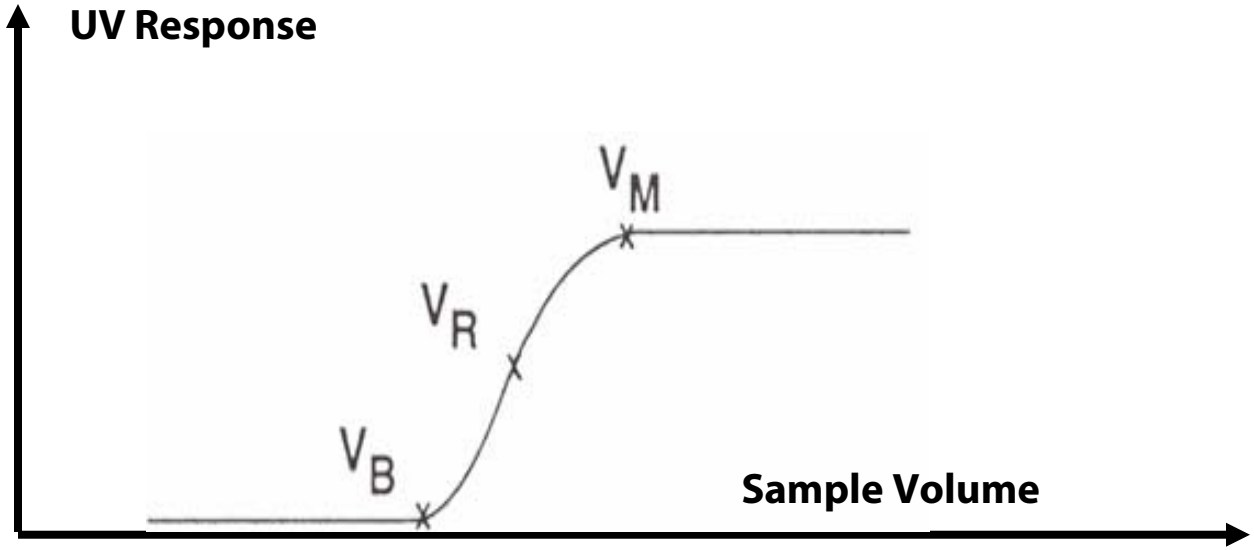
Movement and Fractionation on the Automated System, Access to the Individual SPE cartridge

Fractionation Task





Breakthrough Curve for SPE Device

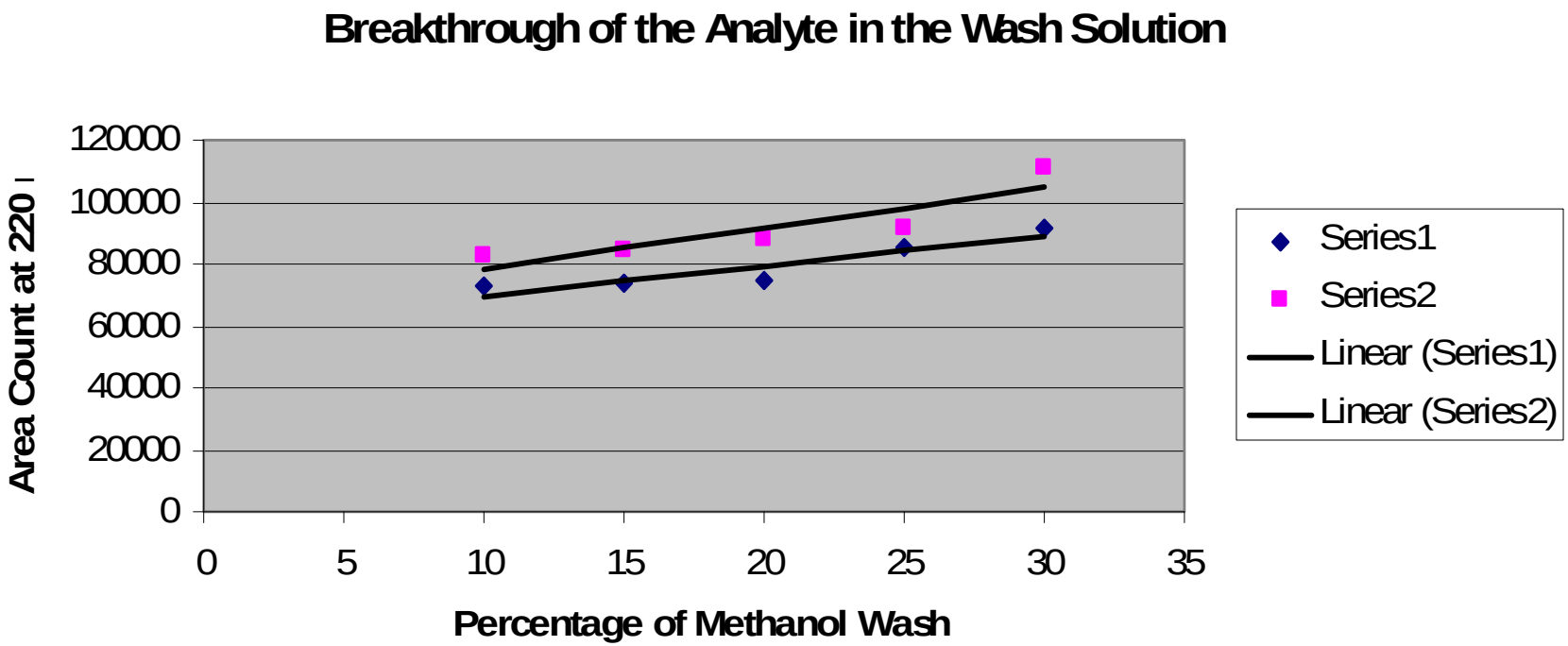


V_B = The volume where analyte first appears (~ 1% maximal value)

V_R = The retention volume

V_M = The volume where maximal analyte breakthrough has occurred (~ 99% of maximal value)

Data and Results:

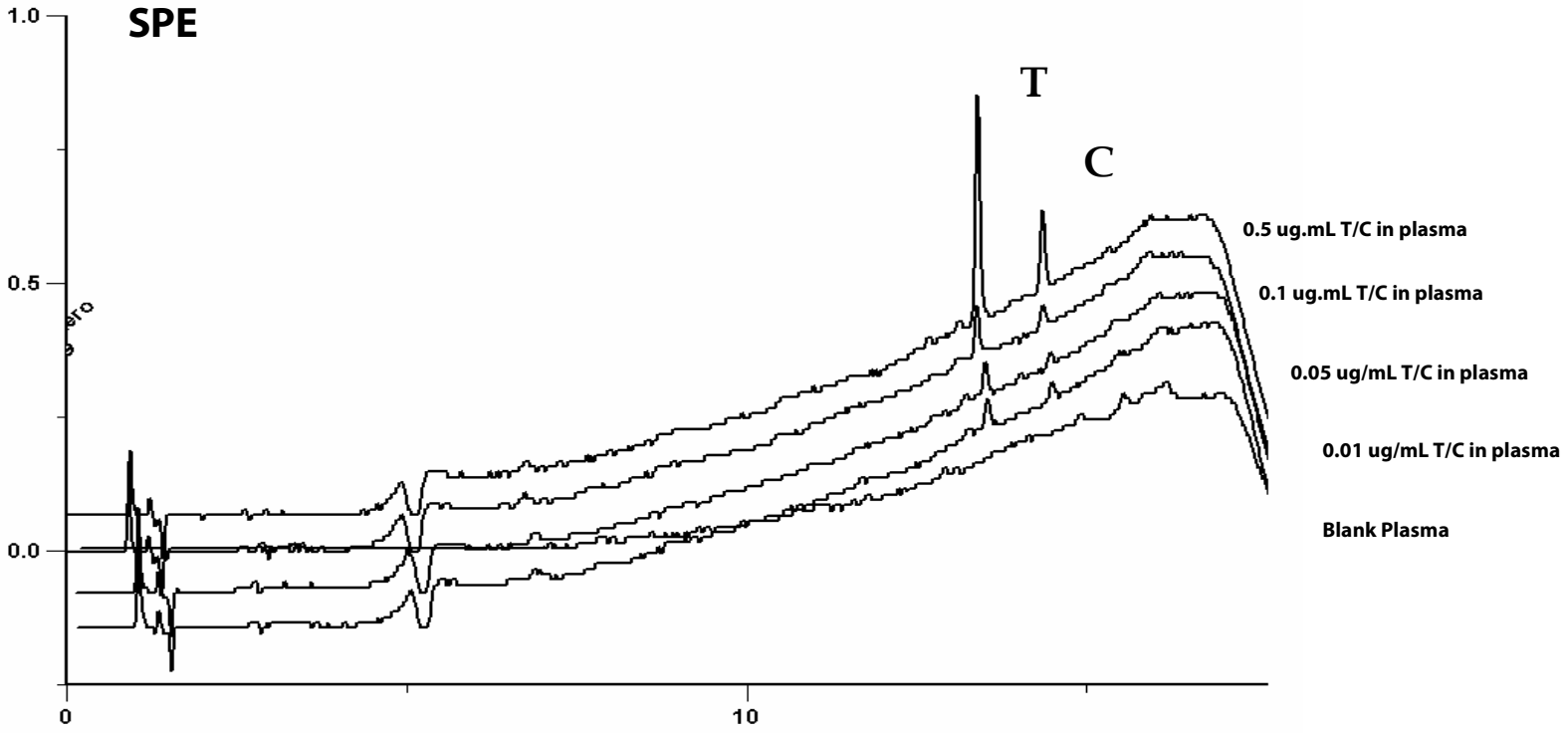


Graph 2: Optimization of the wash solution relative to recovery for Chlorpromazine and Thioridazine at various concentrations of methanol used in the wash solution

Data:

The following data represents the lower levels evaluated

20 ul injection of Spiked Plasma after SPE



Data and Results:

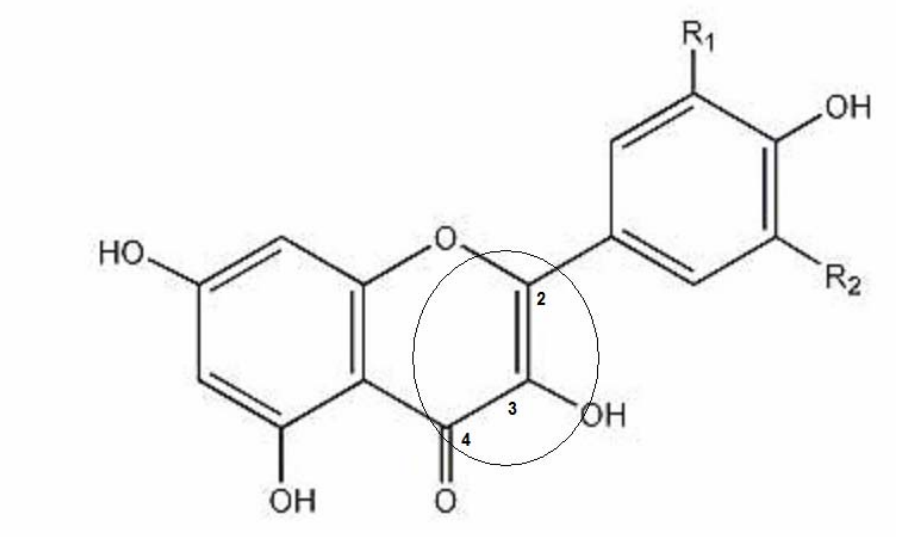
- Changing the elution ratio did not have any distinguishable effect on the recovery
- The recovery of Chlorpromazine and Thioridazine under optimized SPE conditions (15% methanol wash and 2:2:1 elution buffer) yielded 99% and 98%, respectively
- Although the increase in recovery for Chlorpromazine is negligible the increase in recovery for Thioridazine is significant ~10%
- A good rule of thumb is that SPE cartridges retain a mass of solute (analyte plus retained contaminants) this is equivalent to 5% of the sorbent mass
- Therefore using an automated optimization SPE system yielded positive results without manual intervention

Flavonoids

- Found in plant material and products produced from plant material
- Anthocyanins wide color range
  - Blue to red
- Produced by plants for environmental protection
  - UV protection in the 280 – 380 nm range
  - Microorganism infestation protection by phytoalexins
  - Host specific signal compounds

Classification of Flavonoids

Flavonols



Flavonol	R1	R2
Quercetin	OH	H
Kaempferol	H	H
Myricetin	OH	OH
Isorhamnetin	OMe	H

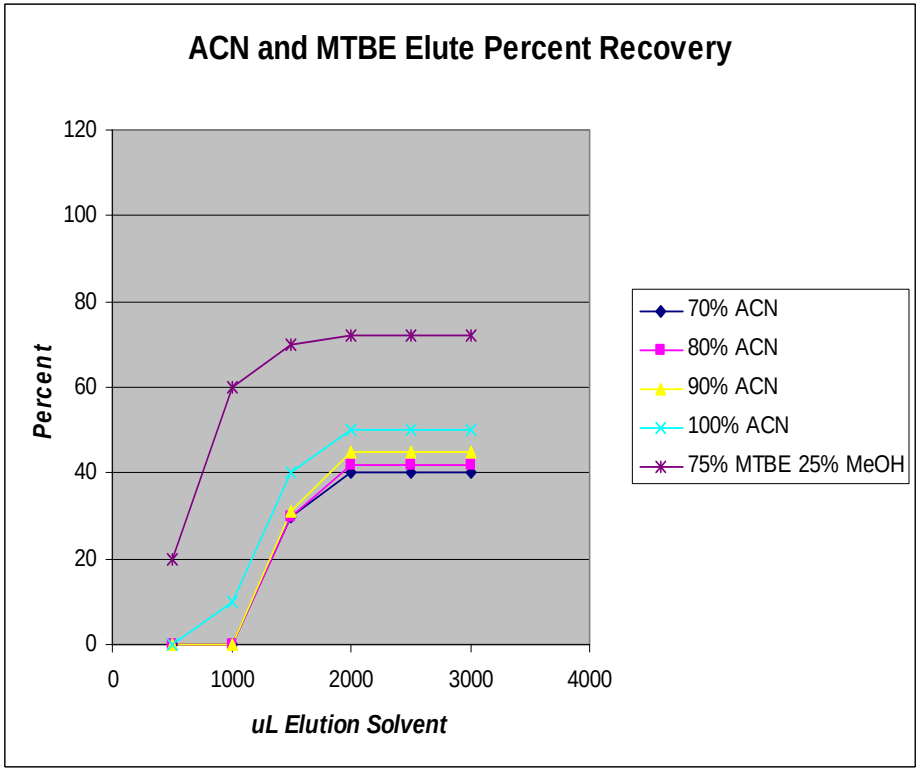
Wash Break-Through

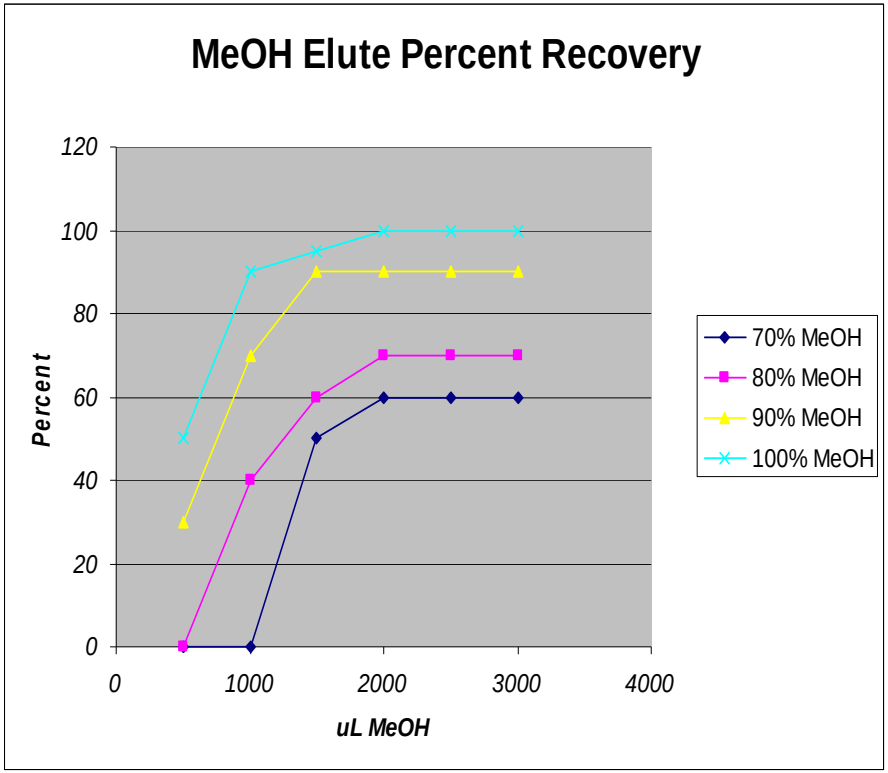
Wash	MeOH			
Break	25	30	35	40
500				
1000				
1500				
2000				
2500				X
3000		X	X	
3500	X			
4000				

MeOH NH4OAc			
25	30	35	40
			X
		X	
	X		
X			

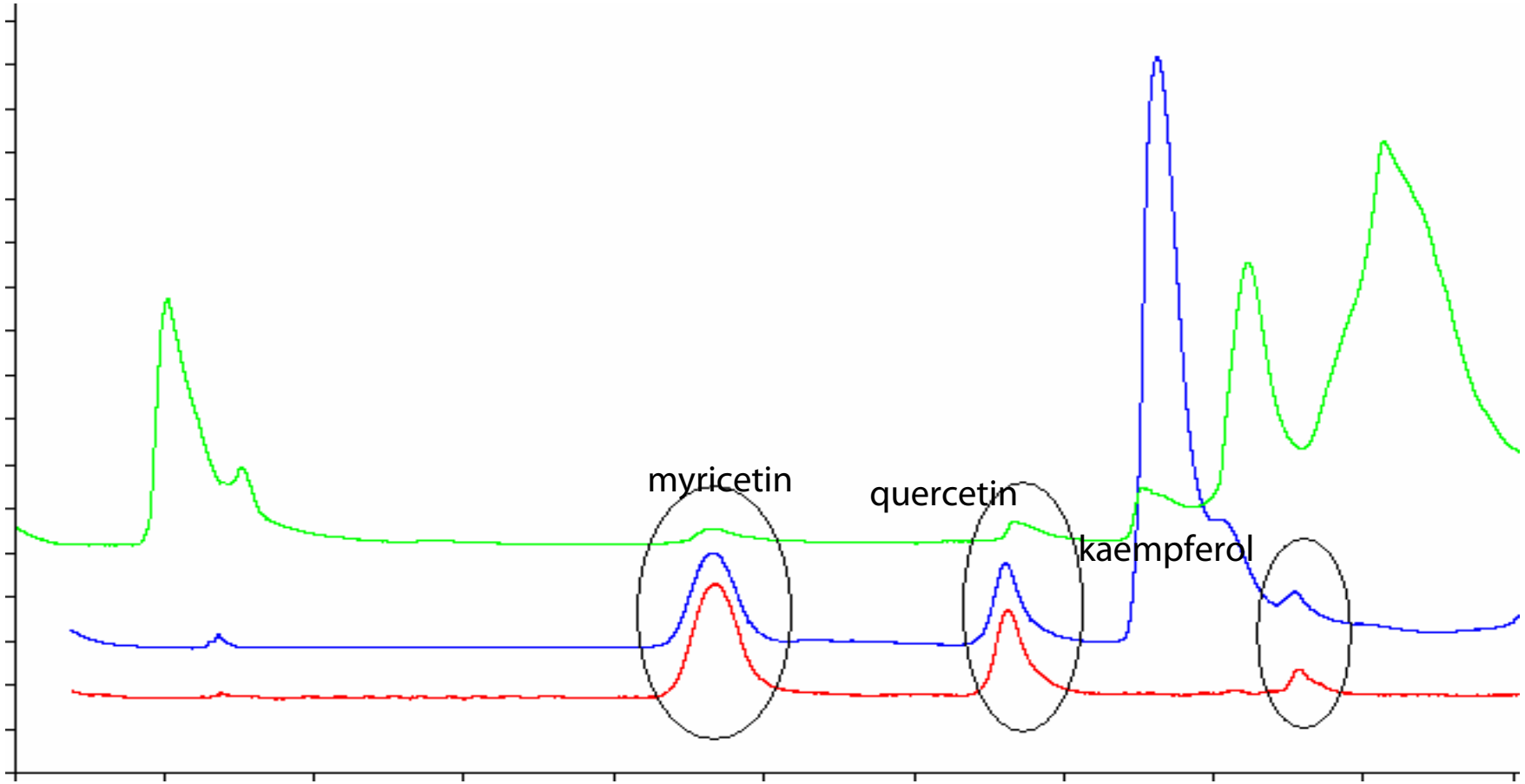
MeOH HCl			
25	30	35	40
		X	X

Elution Percent Recovery





Plasma



Optimization of SPE Method:

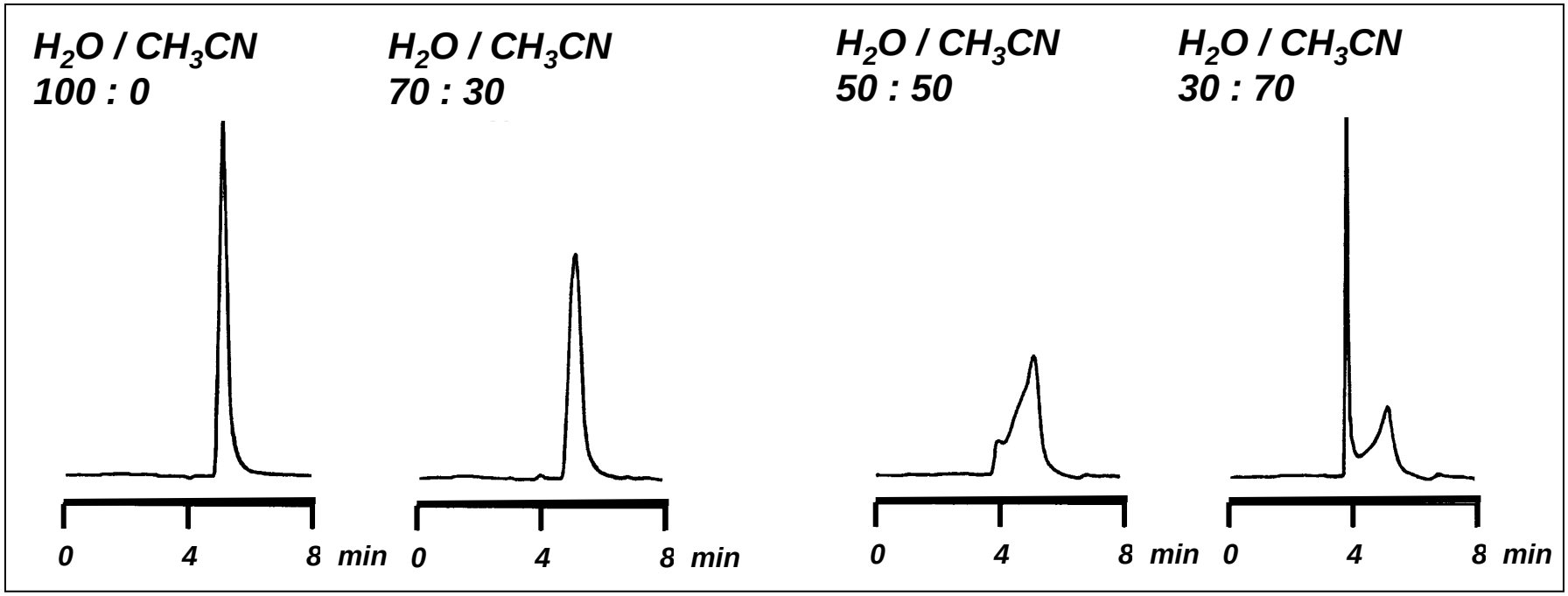
- Flavonoids recovery at 99±1% on all analytes versus ≤ 80 % for quercetin
- SPE of all matrices eliminates interfering peaks
- Optimization of SPE method for multiple matrices allows ease of use when analyzing 6 separate matrices.

Elution Volume:

- Although we've improved the SPE method the story is not finished

- In almost every situation the elution volume is too large for consistent detection S/N or the organic concentration is too high for chromatography analysis

Strong Sample Solvents



The sample should whenever possible be dissolved in the mobile phase

If the sample is dissolved in too strong solvent, significant anomalies occur, peak splitting

Elution Solutions:

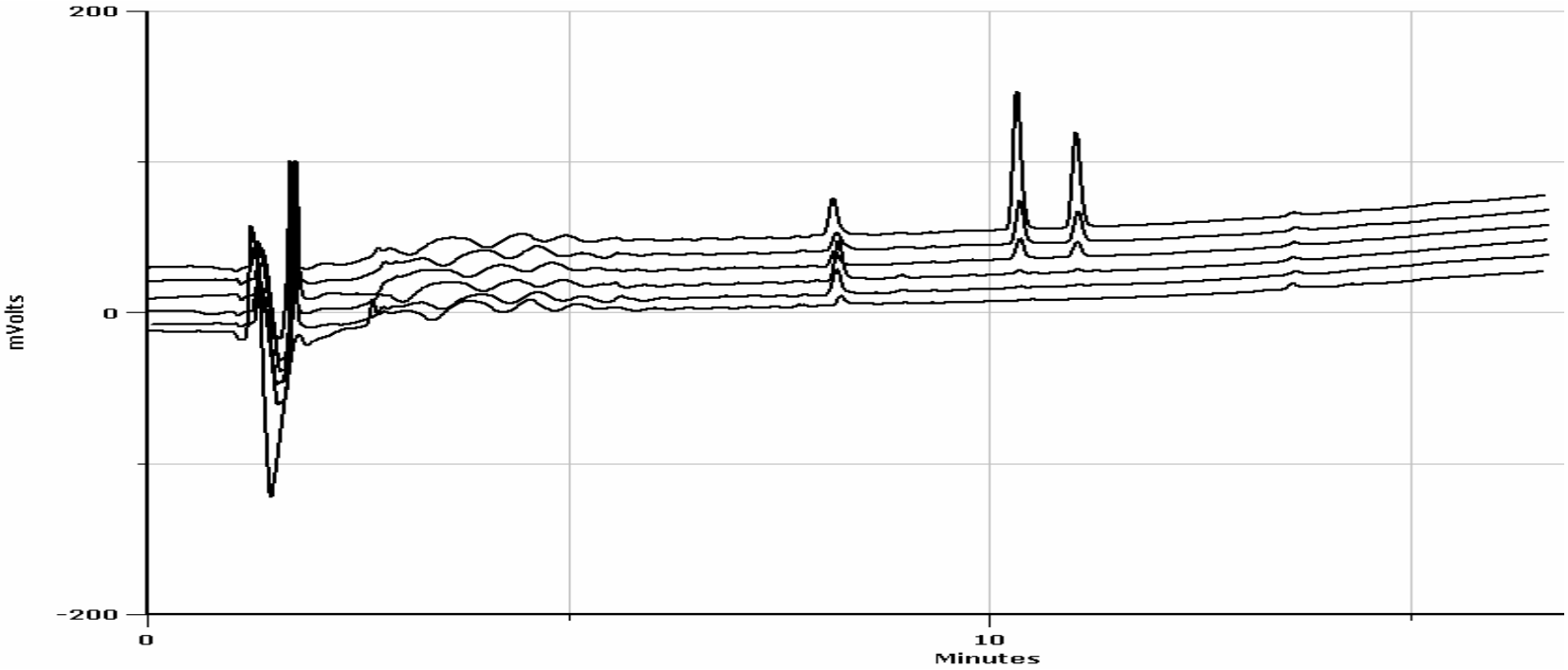
- Drying down the eluent volume either to the point of dryness or to a consistent volume is a major bottleneck in the SPE process, it takes time to dry the samples down and usually involve solubilizing the sample prior to analysis

- Degradation of samples via heating is a concern where the compounds may undergo hydrolysis, esters, which could lead to incorrect interpretation of the results

Alternative Solution:

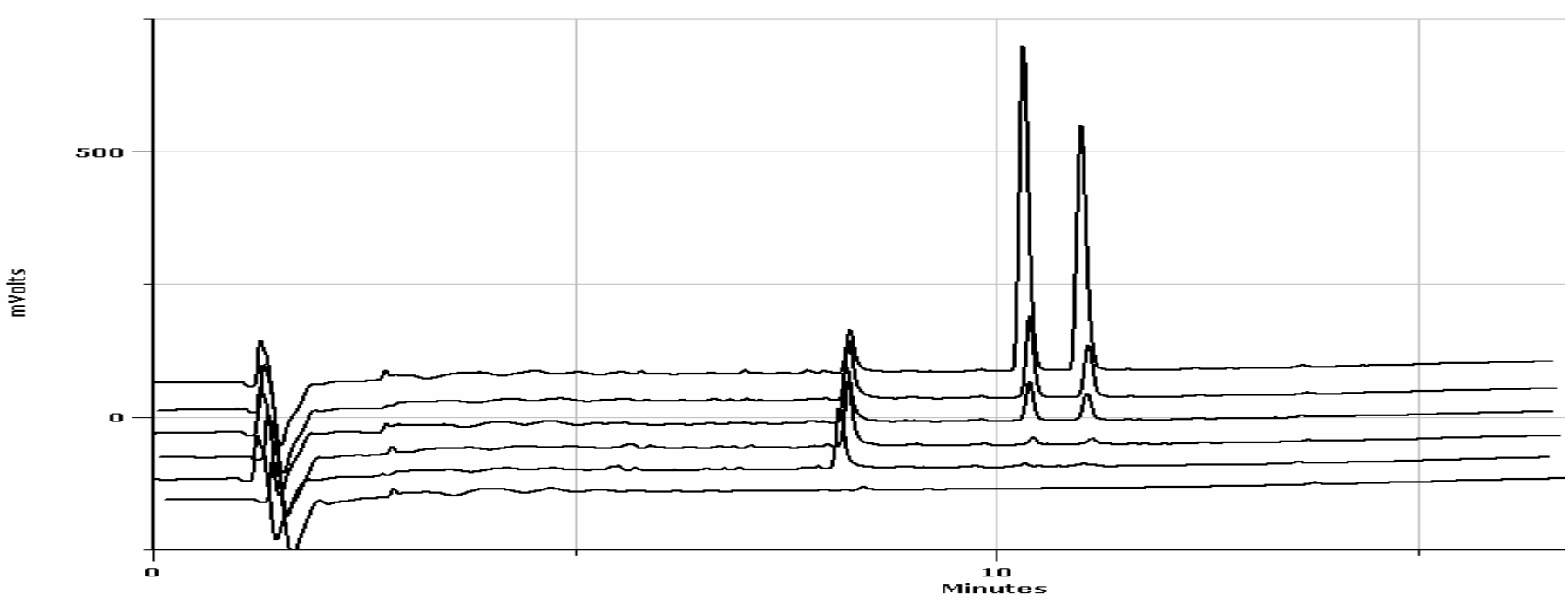
- TEC: trace enrichment cartridges in place of the injection loop
- The eluent from SPE cleanup is diluted with a consistent amount of aqueous mobile phase significantly decreasing the organic strength of the elution solvent
- This diluted eluent solution is then exposed to the TEC
- The TEC will absorb the analyte of interest while the solution will pass though to waste
- The TEC concentrates the analyte and then the TEC is brought inline to the HPLC system
- The TEC not only concentrates the analyte of interest but it removes chromatographic anomalies associated with high organic percentages

Data and Results for TEC:



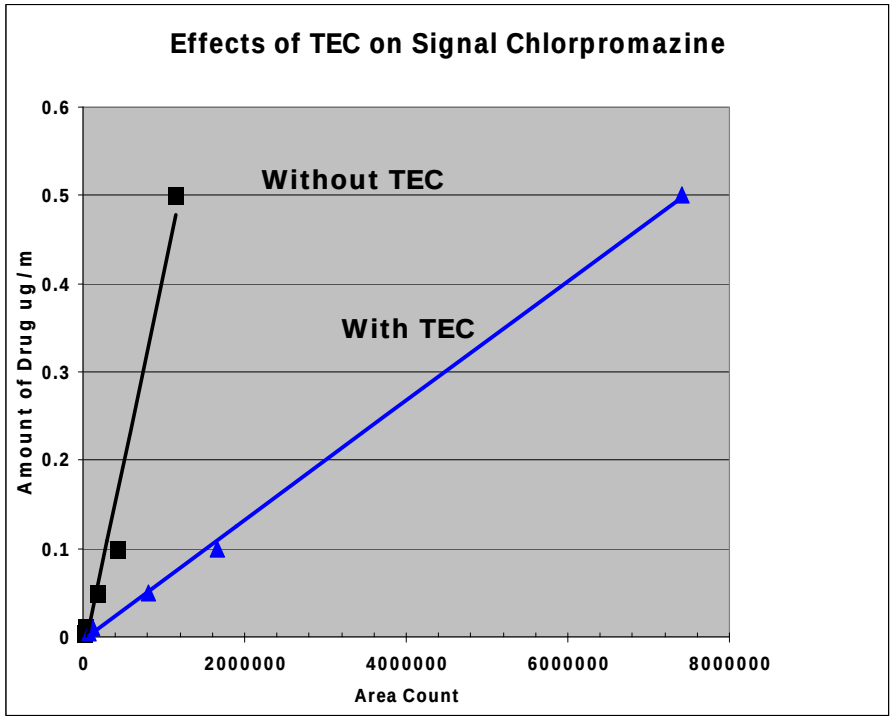
Graph 3: Overlay of the plasma samples at various concentrations of Chlorpromazine and Thioridazine after SPE but without TEC (trace enrichment). A 20 ul sample was injected onto the analytical column, 0.5 mg/mL-0.005 mg/mL concentrations.

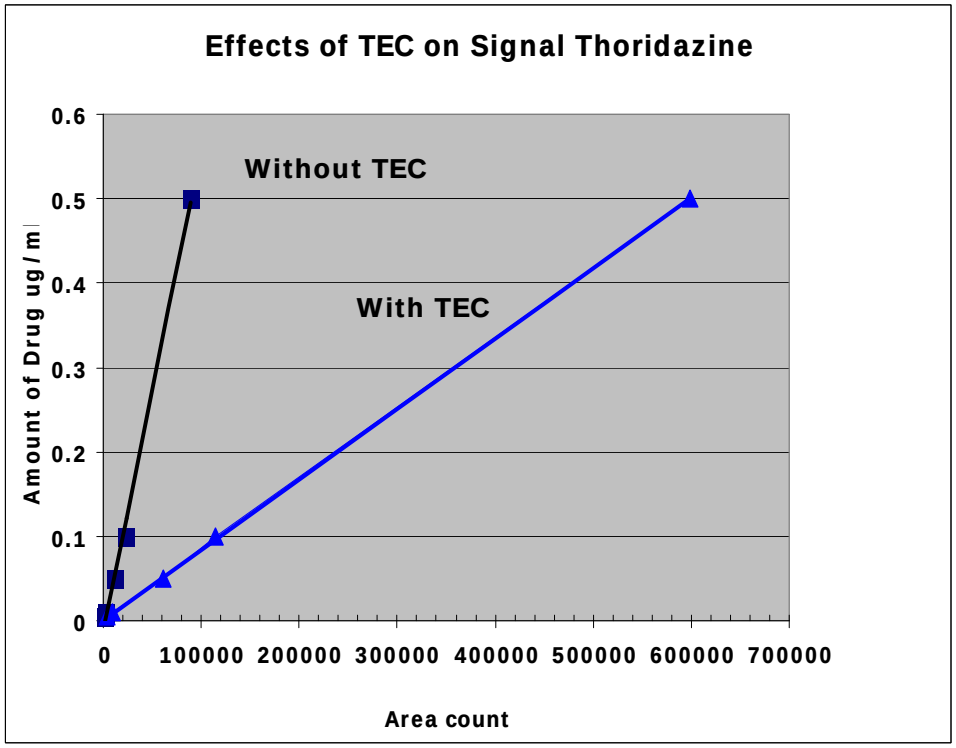
Data and Results for TEC:



Graph 4: Overlays of the plasma samples at various concentrations of Chlorpromazine and Thioridazine. A 900 ul volume injection was introduced to the TEC (trace enrichment) prior to analysis via the analytical column, 0.5 mg/mL- 0.005 mg/mL concentrations.

Data and Results for TEC:





Graphs 5 & 6: Represent the substantial increase in signal when TEC was incorporated prior to the chromatographic analysis. A 900 uL sample was loaded onto the TEC, then the TEC was switched in-line to the analytical column, increasing the S/N by 6 fold.

Conclusion:

- SPE is an important cleanup method for biological and environmental samples
- A good rule of thumb is that SPE cartridges retain a mass of solute (analyte plus retained contaminants) this is equivalent to 5% of the sorbent mass
- Optimizing SPE methods can be very advantageous however extremely labor intense and time consuming
- The automated Liquid Handler and SPE racks allow for the optimization process to take place via multiple collection sites and various conditions without manual intervention
- Incorporating the use of a TEC instead of drying down the eluent from SPE cleanup also offers a very advantageous alternative removing the dry down bottleneck and analyte degradation

THANK YOU!