Updating Solid Phase Extraction Methods: Tips and Tricks for Improving Existing and New SPE Methods using Method Development

Golnar Javadi

Application Engineer

Sample Preparation Products

Spp-support@agilent.com

Today's Agenda

- Updating Existing SPE methods- Why?
- How to Approach SPE Method Development/Optimization?
- Example 1: Solid Phase Extraction of Phencyclidine (PCP) in Urine by Mixed Mode-Bond Elut Certify
- Example 2: Solid Phase Extraction of Opiates in Urine by Mixed Mode-Bond Elut Certify
- Summary
- Questions and Wrap Up

UPDATING EXISTING SPE METHODS- WHY?

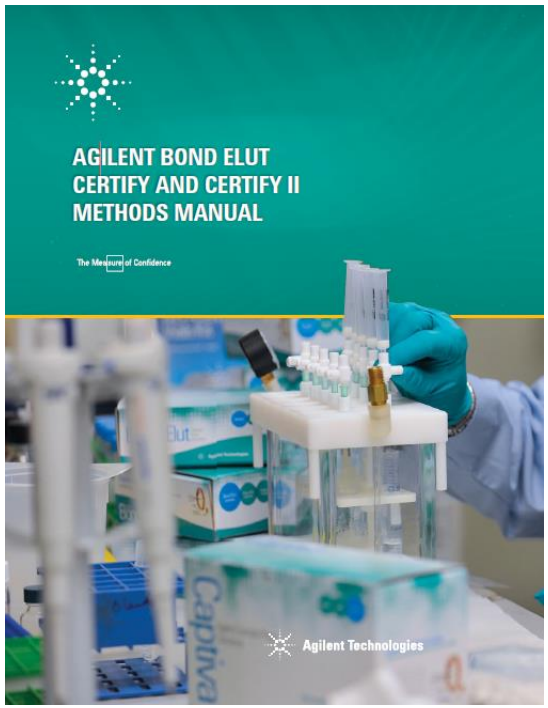
The Need for Updating Existing SPE Methods

- Requirements for lower detection limits
- Improved linearity in the lower or upper concentration range and calibration range extension
- Limited sample volume
- To improve the method to get cleaner samples in order to protect the high end analytical instruments and columns from sample contamination

Bond Elut Certify

- Bond Elut Certify, Mixed Mode (nonpolar + SCX)
- Bond Elut Certify II, Mixed Mode (nonpolar + SAX)

Certify Methods Manual

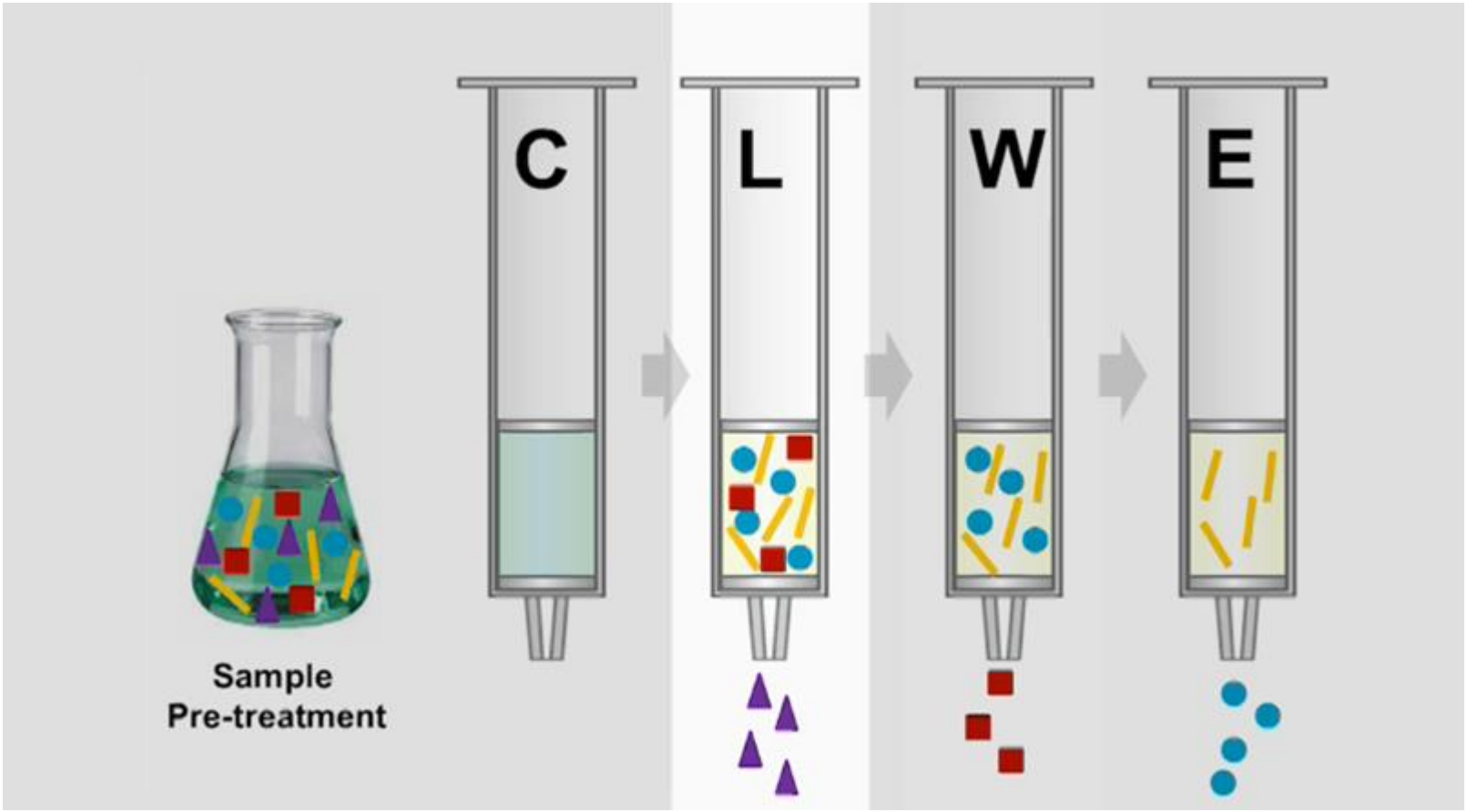


HOW TO APPROACH SPE METHOD DEVELOPMENT/OPTIMIZATION?

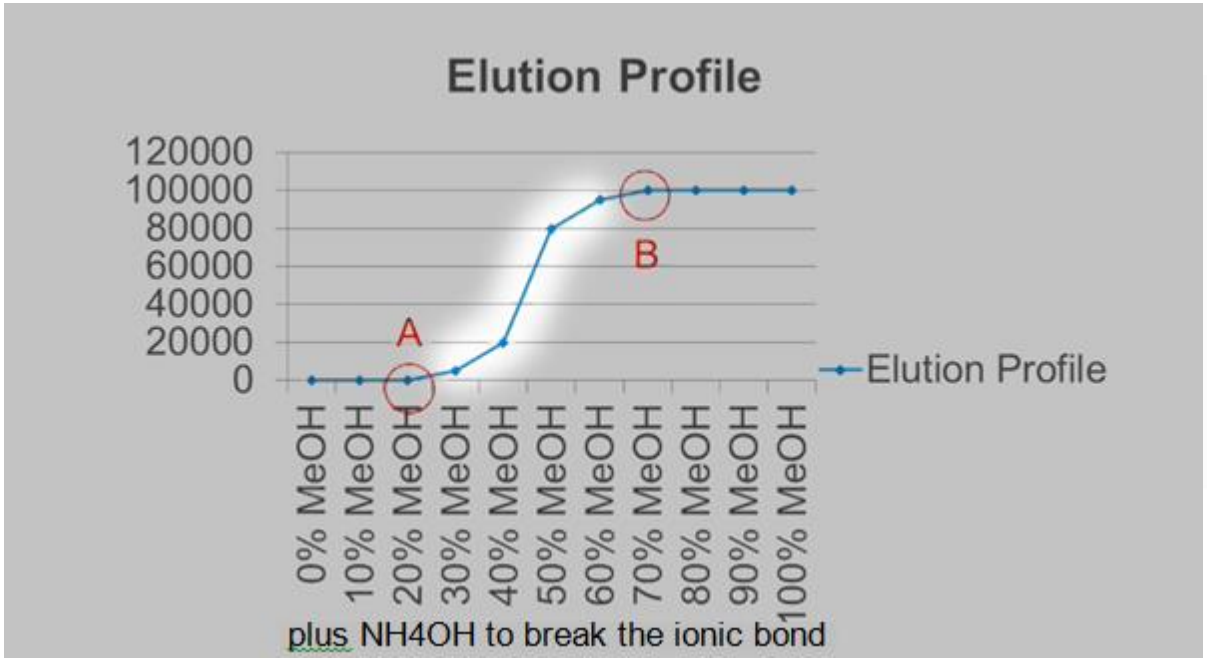
Solid Phase Extraction Sorbents

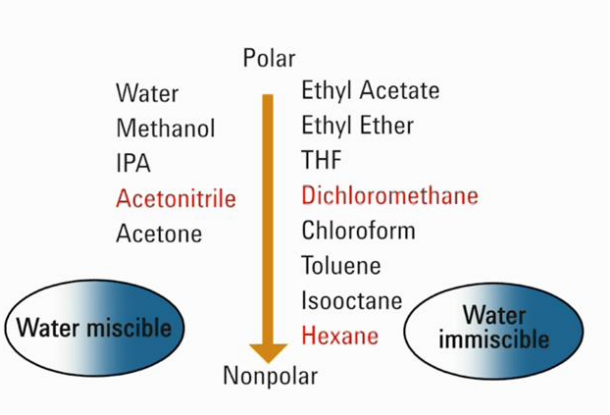
- Nonpolar
- Polar
- Cation exchange
- Anion exchange
- Mixed mode
- Specialty phases

Four Steps of Solid Phase Extraction



10 Bottle Optimization



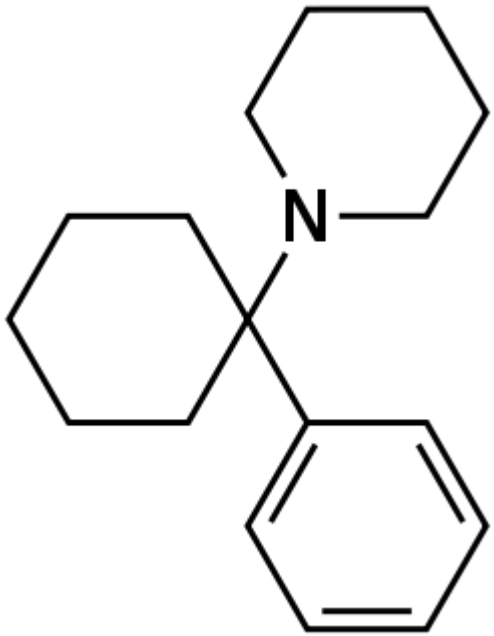


EXAMPLE 1:
SOLID PHASE EXTRACTION OF PCP IN URINE BY MIXED MODE BOND ELUT CERTIFY

Target compound: Phencyclidine (PCP)

pKa: 8.29

Log P: 4.69



PCP: Sample pre-treatment

- Spiked urine pre-treatment:
  - Spike 50 uL of ISTD and STD to 5 mL of urine to have concentration in urine of 100 and 50 ng/mL for PCP and PCP-D5, respectively
  - Add 2 mL of 100 mM KH_2PO_4 (pH = 5.81)

- Blank urine pretreatment:
  - Add 100 uL of water to 5 mL of urine
  - Add 2 mL of 100 mM KH_2PO_4 (pH = 5.81)

- STD sample preparation:
  - Spike 50 uL of ISTD and STD to 7 mL of ACN

PCP:SPE Protocol (BE Certify 130 mg, 3 mL, Part number 12102051)- investigation of wash and elution solvents

Condition: 2 mL MeOH

Equilibrate: 2 mL 100 mM KH_2PO_4

Load: 1 mL of pretreated urine sample (spiked, blank)

Wash 1: 1 mL 5% acetic acid

Wash 2: 1 mL MeoH

Wash 3: 1 mL MeOH

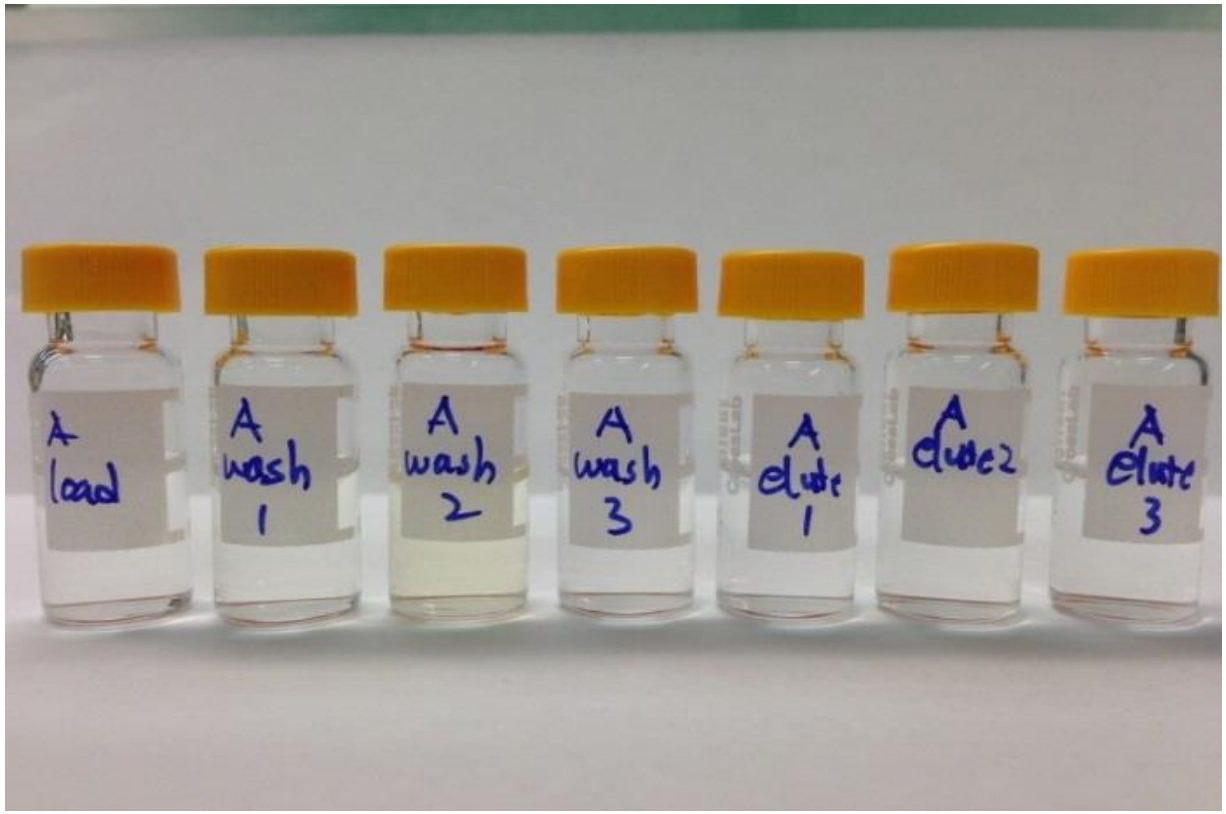
Elute1,2,3: 3 mL ACN + 2% NH_4OH (3X1 mL aliquots, collect each aliquot separately)

Evaporate under nitrogen @ 35 C

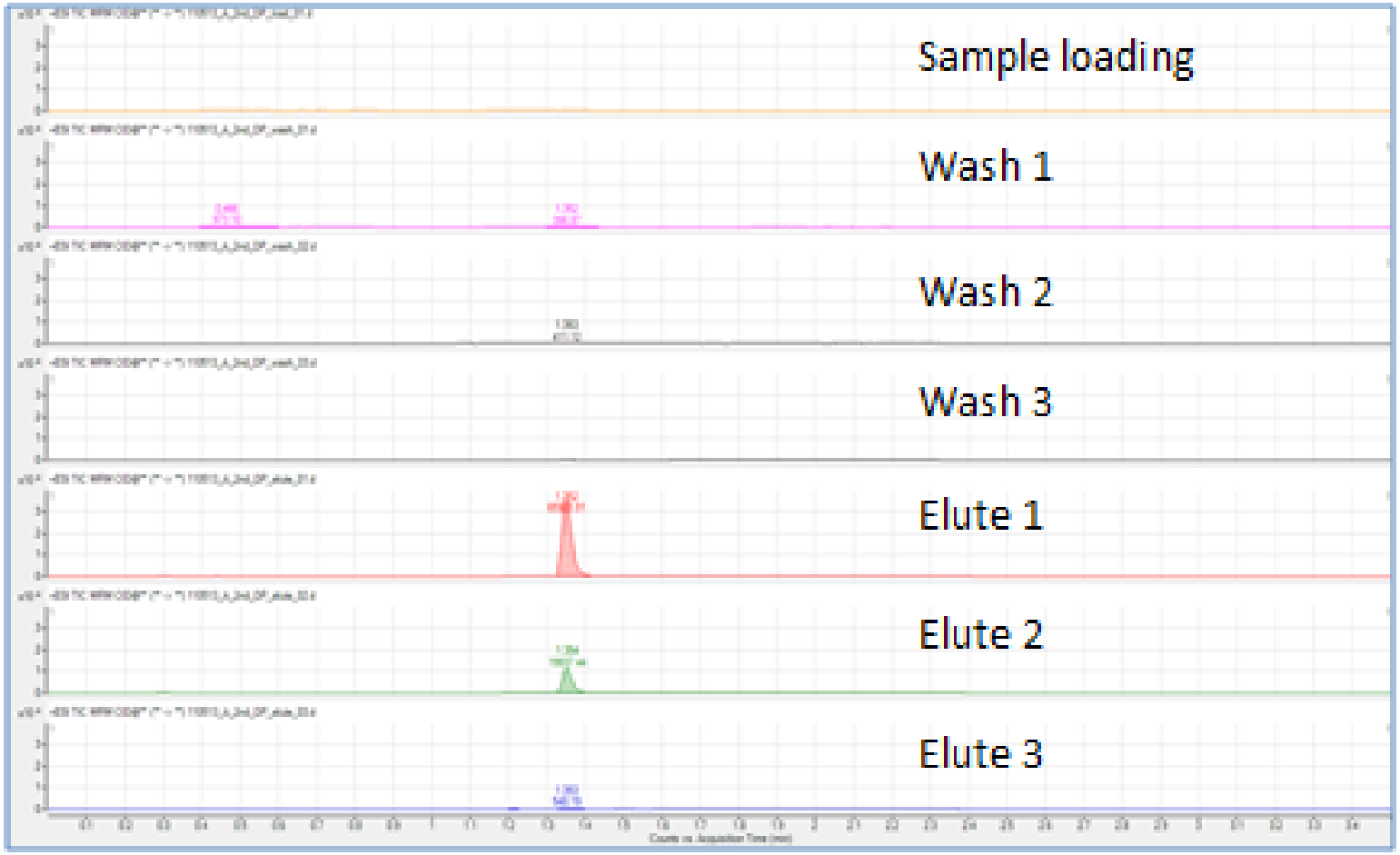
Reconstitute in 0.5 mL EtOAc and 0.5 mL of 10% MeOH for GC and LC samples, respectively

For solvent STD sample, transfer 1 mL of STD mix in ACN to autosampler vials → Evaporate → Reconstitute in 0.5 mL EtOAc and 0.5 mL of 10% MeOH for GC and LC samples, respectively.

PCP: Effluents of load, wash and elution steps



PCP: Chromatograms by LC-MS/MS for all eluates collected during SPE



PCP: SPE Protocol- Investigating different elution solvents

Take 6 cartridges

Condition: 2 mL MeOH

Equilibrate: 2 mL 100 mM KH_2PO_4

Load: 1 mL of pretreated urine sample (spiked, blank)

Wash 1: 1 mL 5% acetic acid

Wash 2: 2 mL MeOH

For each set of two cartridges use one of the following elution solvents. Use one cartridge for LCQQQ, another for GC-MS

Elution 1: 2 mL DCM/IPA (78/20) + 2% /NH4OH -apply to first 2 cartridges

Elution 2: 2 mL of MeOH + 2% NH4OH- apply to the next 2 cartridges

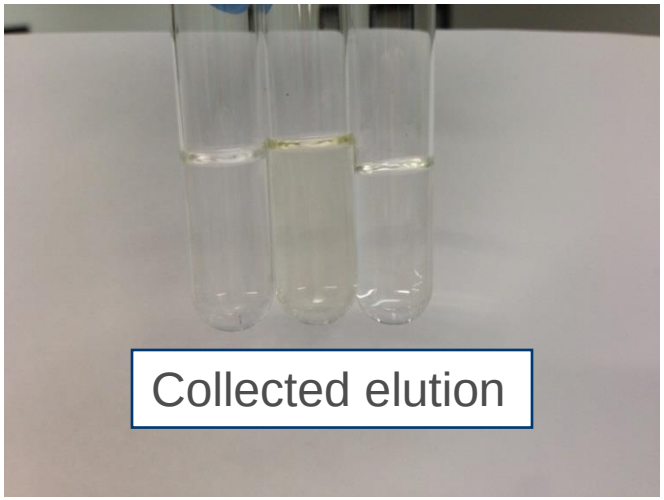
Elution 3: 2 mL of ACN + 2% NH4OH- apply to the last 2 cartridges

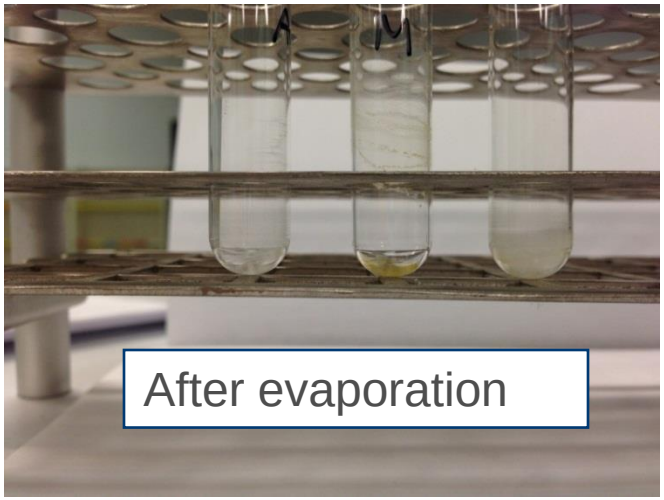
Evaporate under nitrogen @ 35 C

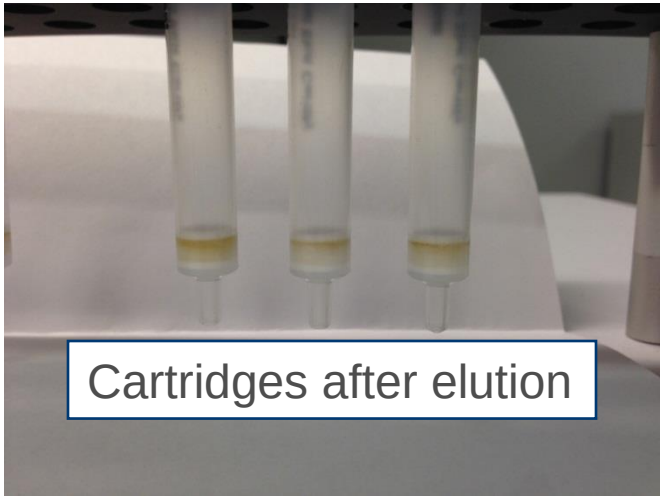
Reconstitute in 0.5 mL EtOAc and 0.5 mL of 10% MeOH for GC and LC samples, respectively

For solvent STD sample, transfer 1 mL of STD mix in ACN to autosampler vials → Evaporate → Reconstitute in 0.5 mL EtOAc and 0.5 mL of 10% MeOH for GC and LC samples, respectively.

PCP: Comparison of Different Elution Solvents







In elution related pictures, from left to right, ACN + 2% NH_4OH, MeOH + 2% NH_4OH, DCM/IPA/NH_4OH 78/20/2 (v/v)

PCP:LC-MS/MS Conditions

Column: Pursuit XRs ULTRA DP 2.8 um, 2.0 X 50 mm

Flow rate: 0.4 mL/min

Gradient:

Time (min)	%B
0	10
1.5	90
2.5	90
2.6	10
5	10

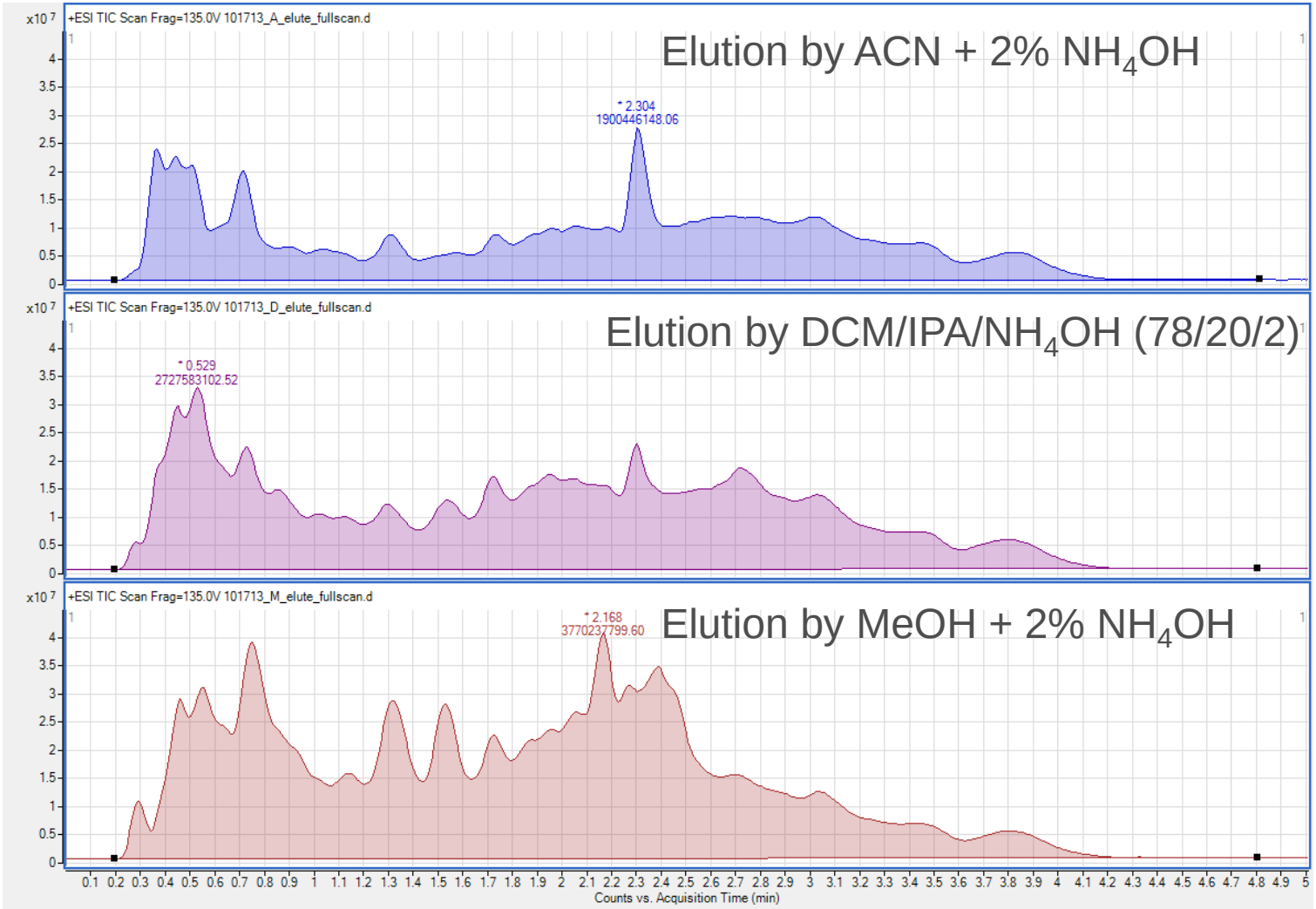
Inj. Vol.: 10 uL

Fullscan: 100 – 550 m/z (positive)

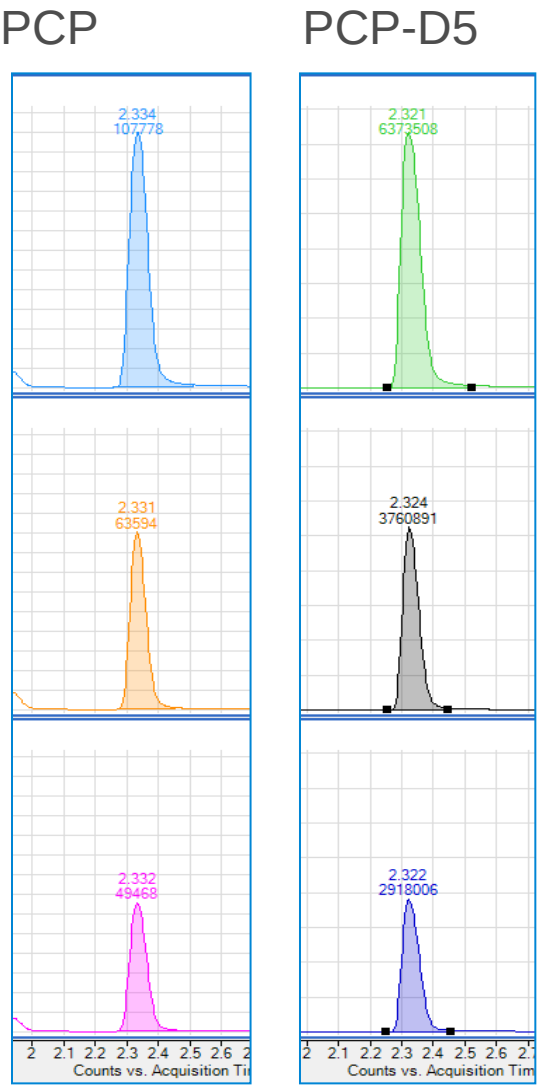
MRM:

	Precursor	Product	MS2 Res	Dwell	Fragmentor	Col Eng	Polarity
PCP-D5	249.2	96.1	Unit	50	97	37	Pos
PCP-D5	249.2	86.1	Unit	50	97	9	Pos
PCP	244.2	91.1	Unit	50	102	37	Pos
PCP	244.2	86.1	Unit	50	102	9	pos

PCP: LC-MS/MS fullscan of blank urine



PCP: LC-MS/MS MRM



→ Elution by ACN + 2% NH_4OH

→ Elution by DCM/IPA/NH_4OH (78/20/2)

→ Elution by MeOH + 2% NH_4OH

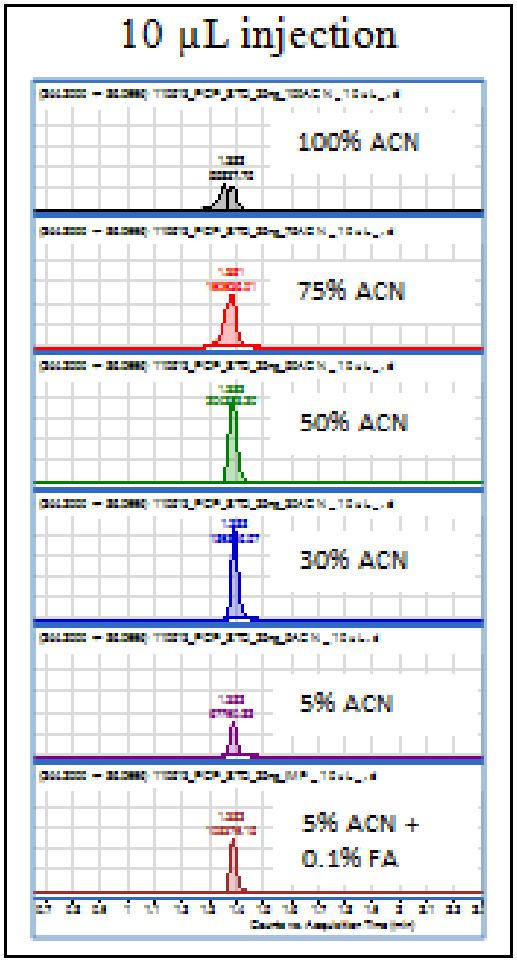
PCP-Final SPE Method after optimization

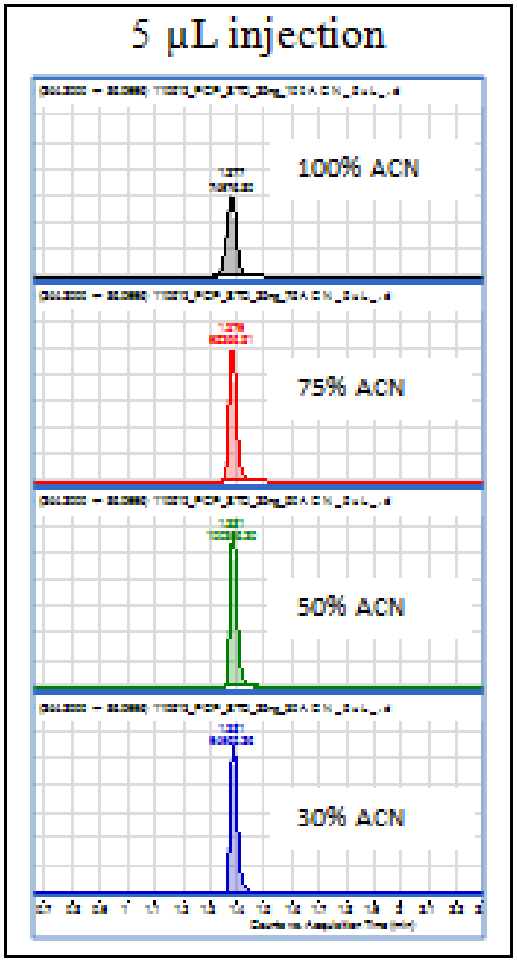
SPE:	Bond Elut Certify, 130 mg, 3 mL, 50/pk (p/n: 12102051)
Condition	1 mL MeOH
Equilibrate	1 mL 100 mM KH_2PO_4
Load:	1 mL of pretreated urine sample (spiked, blank)
Wash 1	1 mL 5% acetic acid
Wash 2	2 mL MeOH
Elute	2 mL ACN + 2% NH_4OH

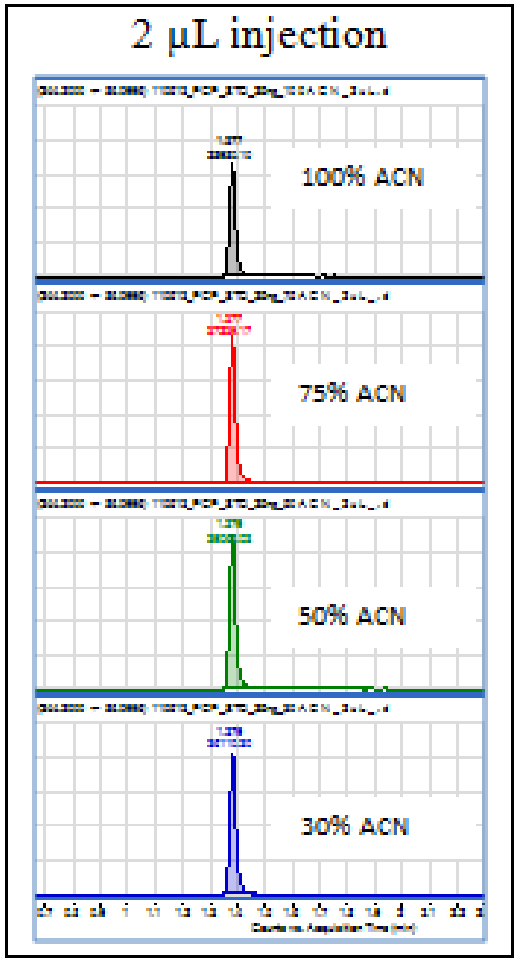
Evaporate and reconstitute in 0.5 mL of 30% ACN + 0.1% FA

(Positive pressure was used for each step.)

PCP: LC-MS/MS Method optimization- Injection volume and injection solvent







PCP: GC-MS Conditions

Column: Agilent DB-5ms UI, 15 m, 0.25 mm, 0.25 um (p/n: 122-5512UI)

Flow rate: 2.5 mL/min

Inj. Vol.: 1 uL

Inlet: S 10:1 (temp: 250 C)

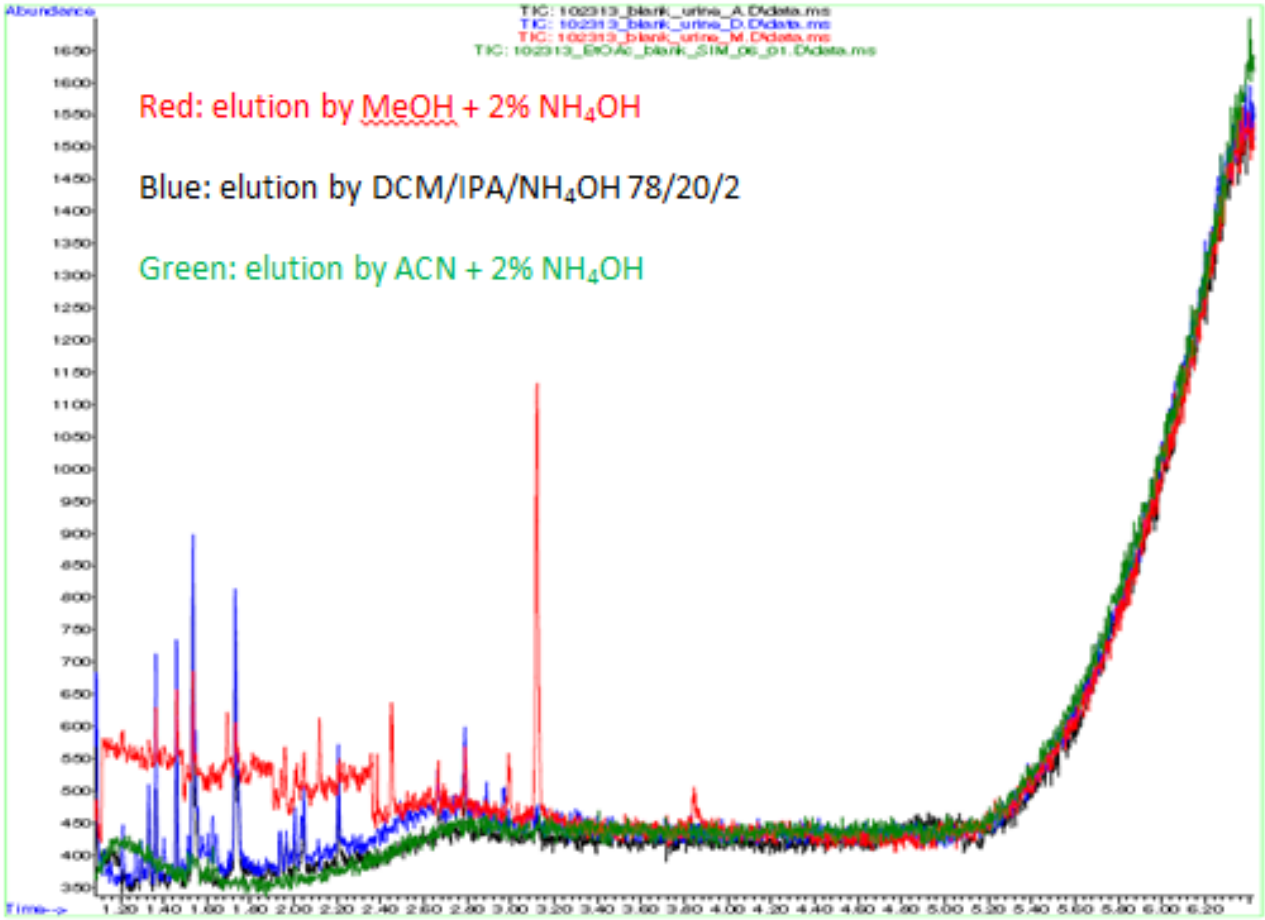
Oven temp: start from 140 C, ramp to 240 C @ 40 C/min, hold for 2.5 min, then to 285 C @ 40 C/min, hold for 0.3 min (6.425 min)

Aux heater: 250 C

MS source: 230 C

MS quad: 150 C

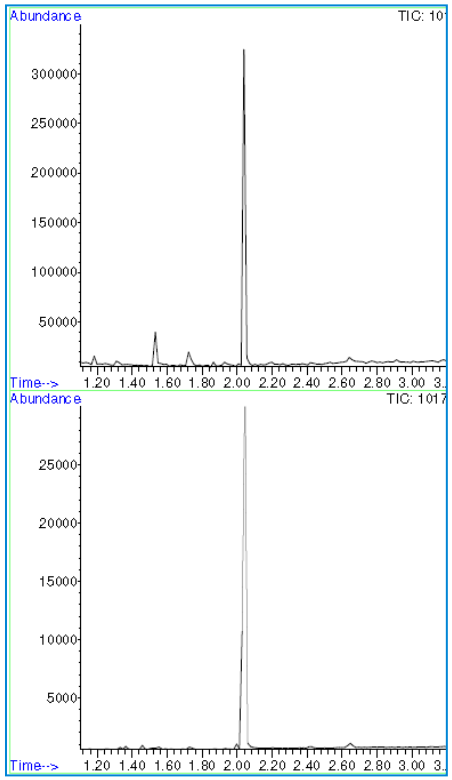
PCP: Fullscan GC-MS chromatograms of blank urine prepared by Bond Elut Certify using different elution solvents



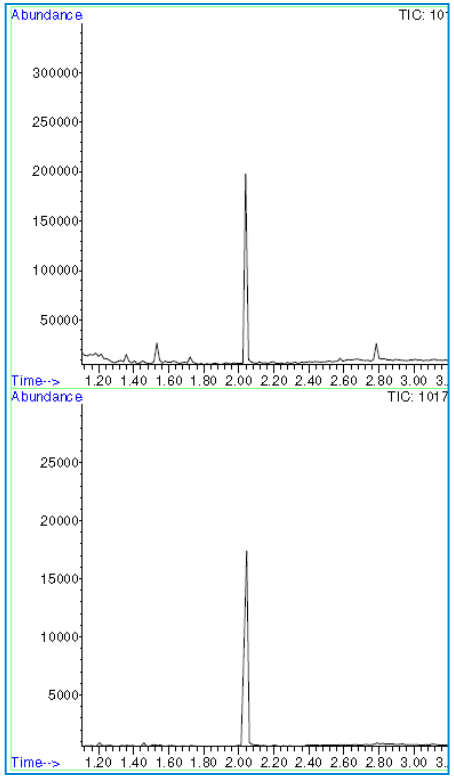
PCP: GC-MS Fullscan/SIM

Fullscan →

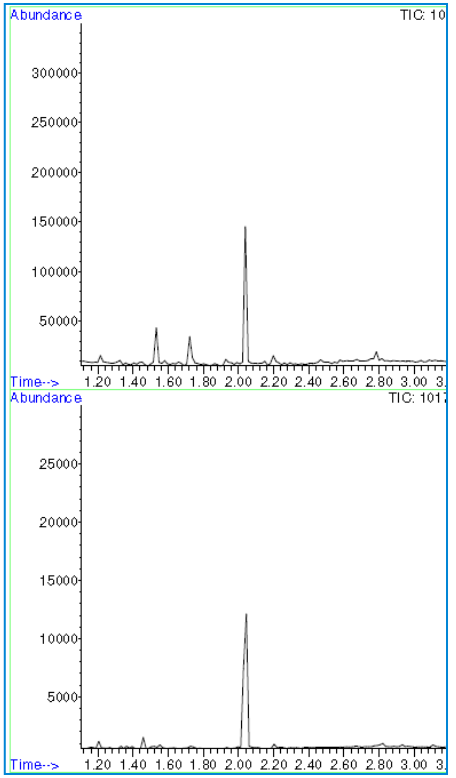
SIM →



Elution by ACN + 2% NH_4OH



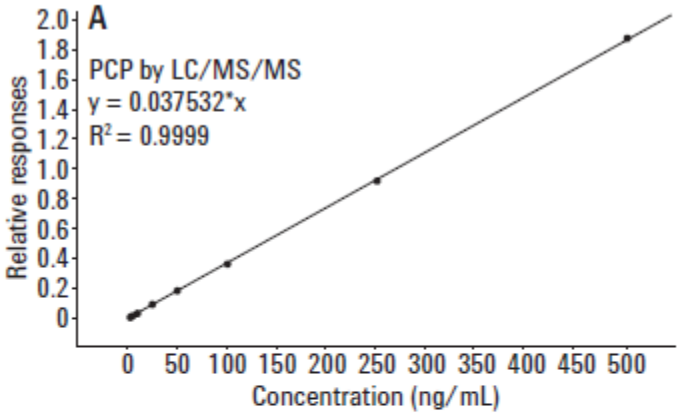
Elution by MeOH + 2% NH_4OH

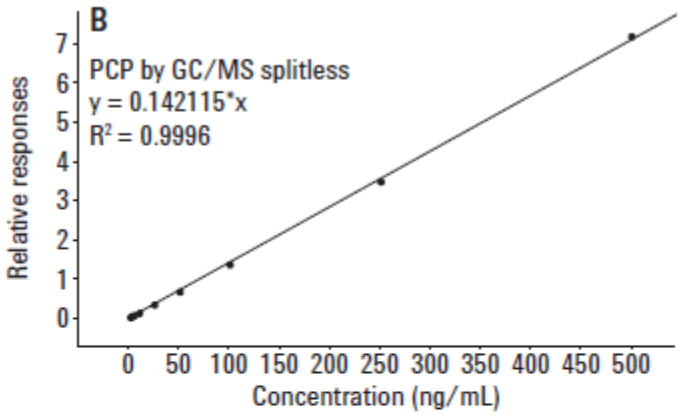


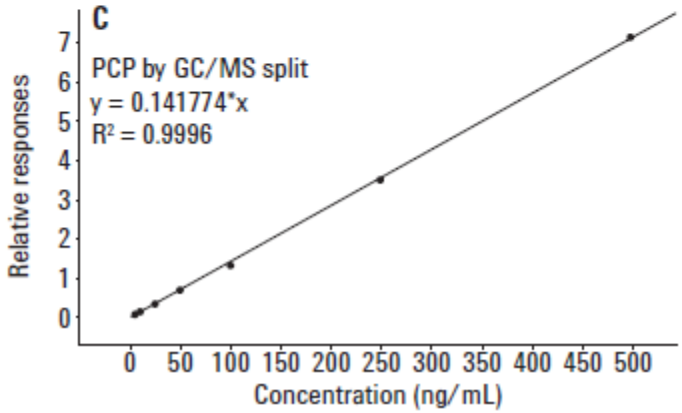
Elution by DCM/IPA/NH_4OH 78/20/2 (v/v)

Y-axis scale is fixed.

PCP: Calibration curves for PCP in urine, 1-500 ng/mL LC-MS/MS and GC-MS







PCP: Summary of LC-MS/MS and GC-MS accuracy and precision data

LC/MS/MS data

R^2	LOQ	Accuracy (% recovery)*(ng/mL)				Precision (% RSD)*(ng/mL)			
0.9999	1 ng/mL	5	10	25	500	5	10	25	500
		101%	92.9%	100%	100%	2.6%	0.7%	0.4%	0.2%

GC/MS data (pulsed splitless injection mode)

R^2	LOQ	Accuracy (% recovery)*(ng/mL)			Precision (% RSD)*(ng/mL)		
0.9996	5 ng/mL	10	25	500	10	25	500
		95.2%	96.0%	97.4%	3.6%	1.6%	2.6%

GC/MS data (pulsed split injection mode)

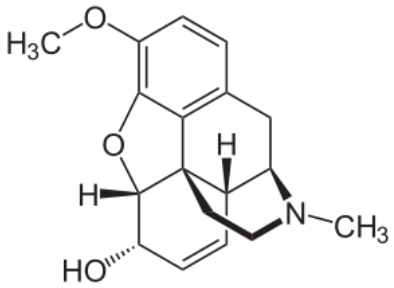
R^2	LOQ	Accuracy (% recovery)*(ng/mL)			Precision (% RSD)*(ng/mL)		
0.9996	5 ng/mL	10	25	500	10	25	500
		101%	99.0%	101%	2.5%	4.4%	3.9%

* Accuracy and precision data are based on six data points.

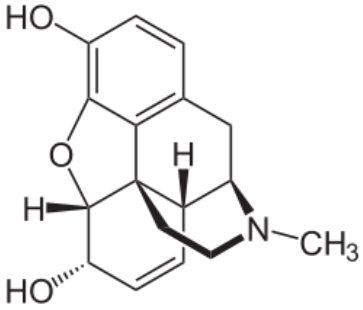
EXAMPLE 2:
SOLID PHASE EXTRACTION OF OPIATES IN URINE BY MIXED MODE-BOND ELUT CERTIFY

Opiates and metabolites

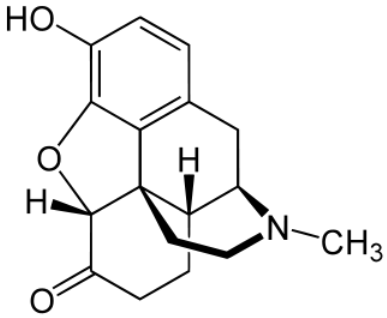
Codeine, pKa 8.2



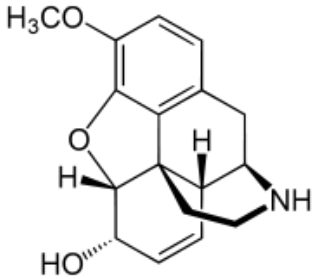
Morphine, pKa 8.2



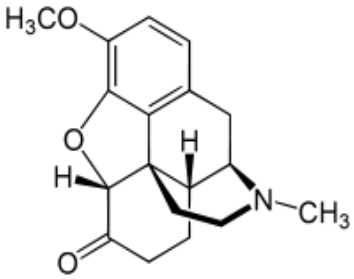
Hydromorphone, pKa 8.6



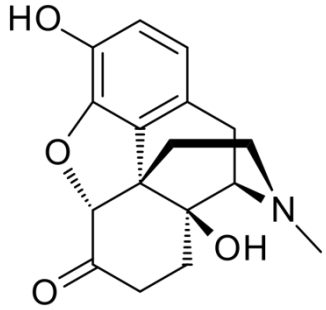
Norcodeine, pKa 9.2



Hydrocodone, pKa 8.6



Oxymorphone, pKa 8.2



Opiates: Sample pre-treatment: acid hydrolysis with pH adjustment after hydrolysis

Acid Hydrolysis – urine spiked with STD, ISTD, INT:

Add 5 mL urine to a 20 mL vial.

Spike 0.5 mL STD, mix.

Spike 0.5 mL ISTD, mix.

Spike 0.5 mL Interference, mix

Add 1 mL concentrated HCl.

Transfer 0.75 mL of spiked urine to 2 mL vials.

Heat at 100 C for 30 min.

Cool down.

Add 0.5 mL 100 mM KH_2PO_4.

Adjust pH with 10 M KOH.

Opiates: double blank urine pre-treatment : acid hydrolysis with pH adjustment after hydrolysis

Acid Hydrolysis – double blank urine:

Add 5 mL urine to a 20 mL vial.

Add 1.5 mL H2O.

Add 1 mL concentrated HCl.

Transfer 0.75 mL of spiked urine to 2 mL vials.

Heat at 100 C for 30 min.

Cool down.

Add 0.5 mL 100 mM KH2PO4.

Adjust pH with 10 M KOH.

Opiates: SPE Protocol - investigation of wash and elution solvents

Condition	1 mL MeOH
Equilibration	1 mL 100 mM KH2PO4
Load	1 mL hydrolyzed urine w/ AND w/o pH adjustment
Wash1	1 mL H2O
Wash2	1 mL 100 mM acetate buffer
Wash3	1 mL MeOH
Wash4	1 mL MeOH
Wash5	1 mL MeOH

For each sample, elute with 3X1 mL aliquots of an elution solvent :

Sample 1		Sample 2		Sample 3	
Elution1	ACN + 2% NH4OH	Elution1	MeOH + 2% NH4OH	Elution1	DCM/IPA:78:20 + 2% NH4OH
Elution2	ACN + 2% NH4OH	Elution2	MeOH + 2% NH4OH	Elution2	DCM/IPA:78:20 + 2% NH4OH
Elution3	ACN + 2% NH4OH	Elution3	MeOH + 2% NH4OH	Elution3	DCM/IPA:78:20 + 2% NH4OH

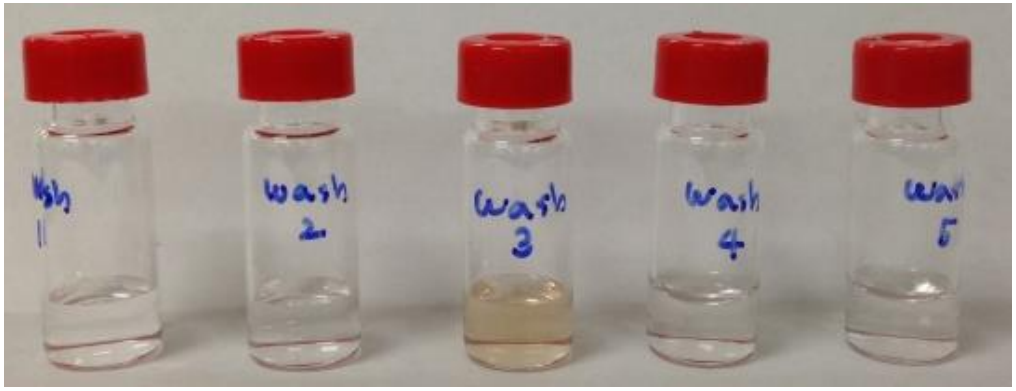
Collect Wash1-5 and Elution1-3 individually.

Evaporate under nitrogen @35C to dryness.

Reconstitute in 0.5 mL 10:90 MeOH+0.1% FA:H2O+0.1% FA.

Run spiked samples by both MRM and fullscan, double blank urine samples by fullscan.

Opiates: Pictures from Washes and Elutions



Wash1	1 mL H2O
Wash2	1 mL acetate buffer
Wash3	1 mL MeOH
Wash4	1 mL MeOH
Wash5	1 mL MeOH

Elution1:1 mL ACN + 2% NH4OH
Elution2:1 mL ACN + 2% NH4OH
Elution3:1 mL ACN + 2% NH4OH

Elution1:1 mL DCM/IPA+ 2% NH4OH
Elution2:1 mL DCM/IPA + 2% NH4OH
Elution3:1 mL DCM/IPA + 2% NH4OH
*DCM/IPA/NH4OH = 78/20/2

Elution1:1 mL MeOH + 2% NH4OH
Elution2:1 mL MeOH + 2% NH4OH
Elution3:1 mL MeOH + 2% NH4OH

Opiates : SPE Method work- in- progress

Condition	1 mL MeOH
Equilibration	1 mL 100 mM KH2PO4
Load	1 mL of hydrolyzed urine sample (pH=6.0 - 6.5)
Wash1	1 mL water
Wash2	1 mL 100 mM acetate buffer
Wash3	2 mL MeOH

Elution:

Elution1	1 mL DCM/IPA/NH4OH:78/20/2
Elution2	1 mL DCM/IPA/NH4OH:78/20/2
Elution3	1 mL DCM/IPA/NH4OH:78/20/2

Collect eluates 1-3 separately
Evaporate under nitrogen @ 35 C to dryness
Reconstitute in 0.5 mL 10:90 MeOH +0.1% FA: H20 + 0.1% FA
Run spiked samples by both MRM and fullscan, double blank urine samples by fullscan

Opiates: investigation of how to apply the elution solvent

Condition	1 mL MeOH
Equilibration	1 mL 100 mM KH2PO4
Load	1 mL of hydrolyzed urine sample (pH=6.0 - 6.5)
Wash1	1 mL water
Wash2	1 mL 100 mM acetate buffer
Wash3	2 mL MeOH

Elution: Try the following three elution methods, collect each eluate separately

Method 1

Elution1	1 mL DCM/IPA/NH4OH:78/20/2
Elution2	1 mL DCM/IPA/NH4OH:78/20/2
Elution3	1 mL DCM/IPA/NH4OH:78/20/2

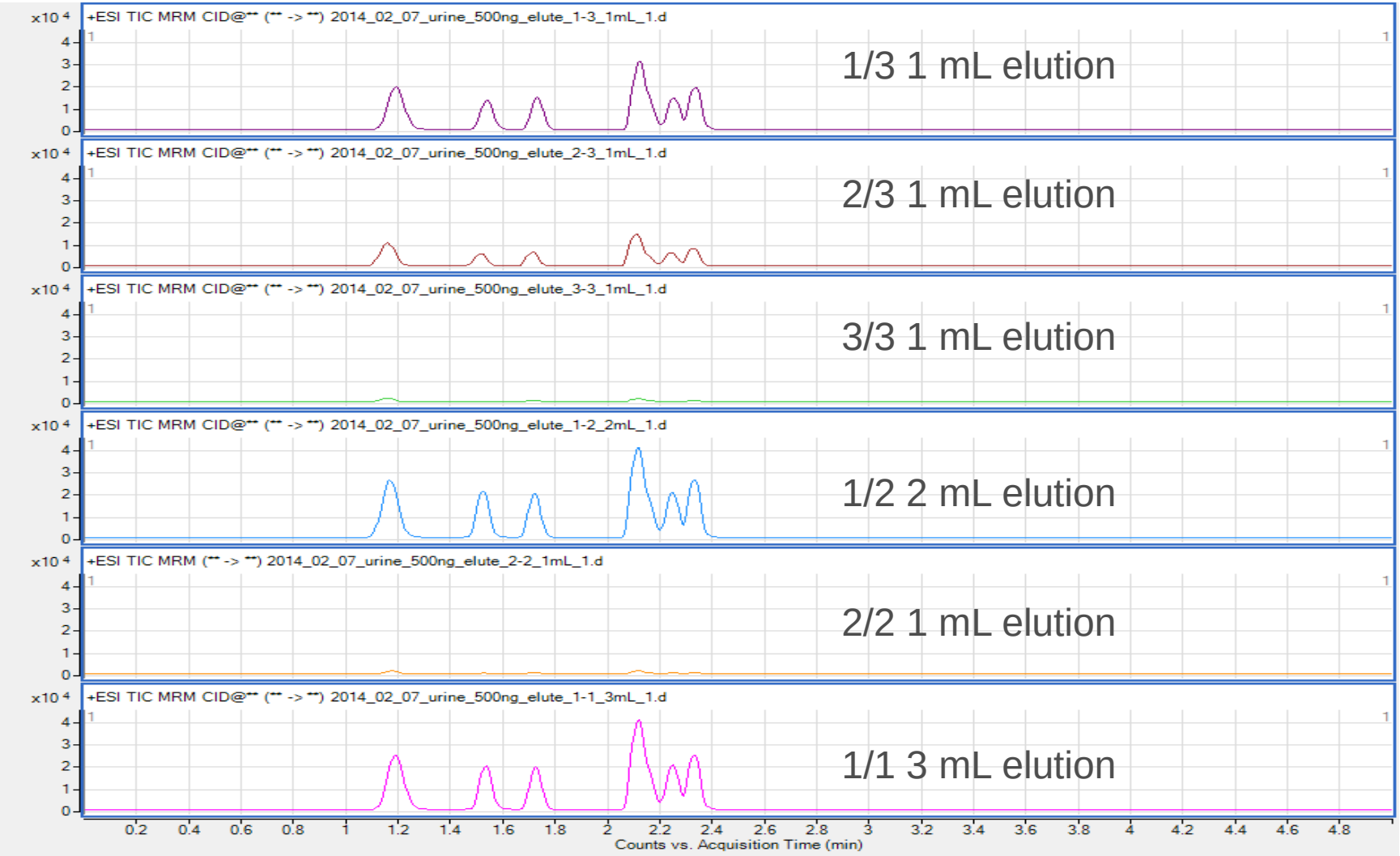
Method 2

Elution1	2 mL DCM/IPA/NH4OH:78/20/2
Elution2	1 mL DCM/IPA/NH4OH:78/20/2

Method 3

Elution1	3 mL DCM/IPA/NH4OH:78/20/2

Opiates: SPE Method - Elution Results



Compounds still coming out after 2 mL of elution solvent → Need stronger elution
e.g. 5% NH4OH

Opiates SPE Method : work- in- progress elution solvent strength study

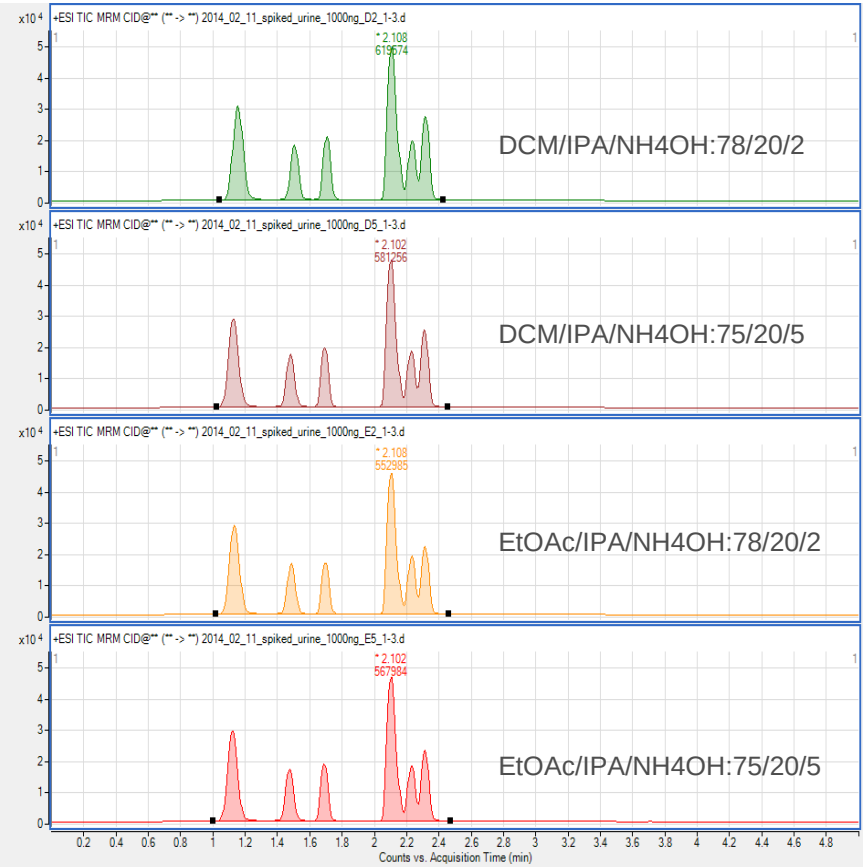
Condition	1 mL MeOH
Equilibration	1 mL 100 mM KH2PO4
Load	1 mL of hydrolyzed urine sample (pH=6.0 - 6.5)
Wash1	1 mL water
Wash2	1 mL 100 mM acetate buffer
Wash3	2 mL MeOH
Elution	4 different elution solvents were used shown as below. 1st, 2nd, 3rd elution were collected separately

Dry under nitrogen at 35C to dryness
Reconstitute in 0.5 mL of 10% MPB.

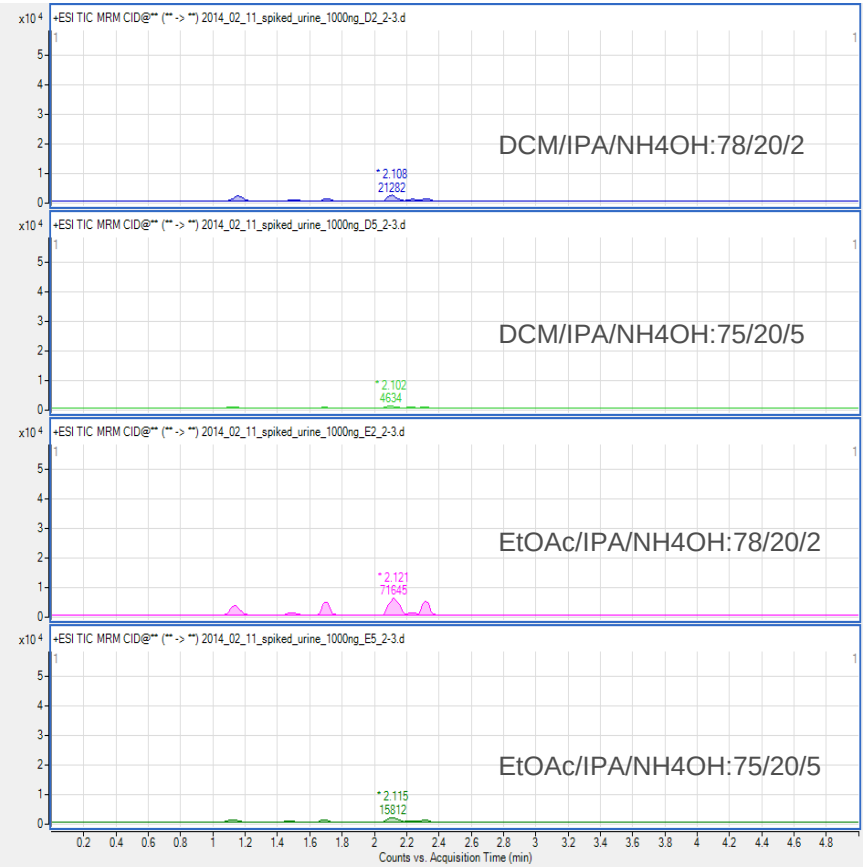
	1st elution	2nd elution	3rd elution
DCM/IPA/NH4OH:78/20/2	2 mL	2 mL	2 mL
EtOAc/IPA/NH4OH:78/20/2	2 mL	2 mL	2 mL
DCM/IPA/NH4OH:75/20/5	2 mL	2 mL	2 mL
EtOAc/IPA/NH4OH:75/20/5	2 mL	2 mL	2 mL

Opiates SPE Method: Elution Study Result

1st elution



2nd elution



- DCM vs. EtOAc → In general, EtOAc had slightly lower elution performance.
- **2% vs 5% NH4OH → Did not show overall performance different, but 2% NH4OH had 2nd elution peaks 4 – 5 % of the 1st elution peaks. So 5% NH4OH is chosen.**

Opiates-Final SPE Method after optimization

Condition	1 mL MeOH
Equilibration	1 mL 100 mM KH_2PO_4
Load	1 mL of hydrolyzed urine sample (pH=6.0 - 6.5)
Wash1	1 mL water
Wash2	1 mL 100 mM acetate buffer
Wash3	2 mL MeOH
Elution	2mL DCM/IPA/NH_4OH:75/20/5

Dry under nitrogen at 35C to dryness

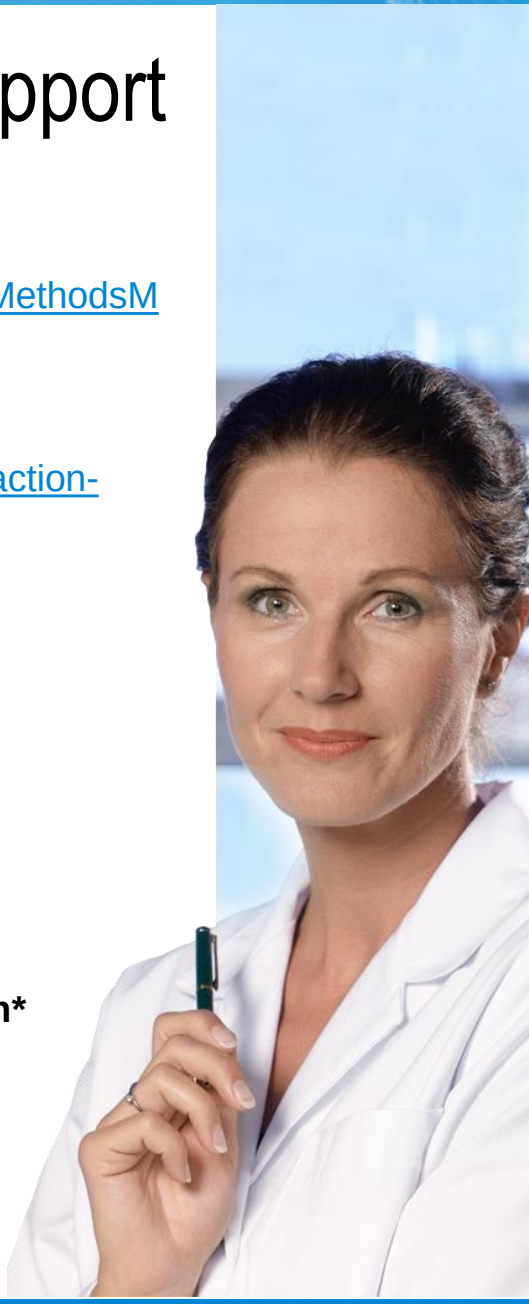
Reconstitute in 0.5 mL of 10% MPB.

Summary and Conclusions

- Systematic approach to method optimization can modify old methods to fit new requirements and different analytical techniques.
- Bond Elut Certify methods can be adjusted to be used with LC-MS/MS as well as GC-MS. The same Certify method can be used with both techniques.
- Bond Elut Certify is an ideal SPE product for analysis of basic drugs from biological samples with great linearity, LOD, accuracy, and precision.
- Many application notes are available in Certify Methods Manual.

Additional Resources and Application Support

- **Bond Elut Certify Methods Manual:** http://www.chem.agilent.com/Library/brochures/Bond%20Elut%20Certify%20MethodsManual.pdf
- **Bond Elut Certify Video:** http://www.chem.agilent.com/en-US/products-services/Columns-Sample-Preparation/Sample-Preparation/Solid-Phase-Extraction-(SPE)/Bond-Elut-Certify/Pages/certifyvideo.aspx
- **Application note 5991-4695EN:** http://www.chem.agilent.com/Library/applications/5991-4695EN.pdf
- **Application note 5991-4575EN**: http://www.chem.agilent.com/Library/applications/5991-4575EN.pdf



Agilent Sample Preparation Products Technical Support Contact Information*

Phone: 800-227-9770, Options 3, 3, 3

Email: spp-support@agilent.com

* *North America*

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Questions?

Thank You

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