Proof of Concept for Automated SPE/HPLC/MS/MS Methods to Replace Traditional Immunoassay with MS Confirmation of Driving Under the Influence Samples

Robert M. Sears, Toxicology Technical Leader[1] , Kenneth Lewis, CEO[2] , and Kim Gamble, President[3]
[1]SC Law Enforcement Division, Columbia, SC 29221
[2]OpAns LLC, Durham, NC 27713, [3]ITSP Solutions, Inc., Suwanee, GA 30024

Introduction

Immunoassay for screening followed by solid phase extraction (SPE) coupled with GC/MS or LC/MS/MS is well established for identification and confirmation/quantification of drugs and/or poisons from complex biological matrices submitted to forensic laboratories. However, reduced budgets and staffing necessitate improved operational efficiency. This poster details our initial comparison of operational efficiency using in-line automated SPE HPLC/MS/MS, versus traditional methods, for the analysis of urine samples submitted in Driving Under the Influence of Drugs (DUI-D) cases.

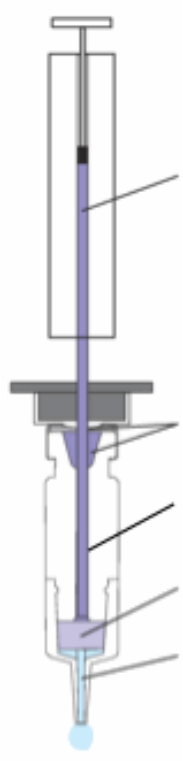
Background

Many clinical/forensic labs face difficulties related to budget cuts, reduced staffing, the need to effectively utilize instrument time and resources, and a need to increase the productivity of the remaining scientists. Instrument Top Sample Preparation (ITSP) coupled to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) provides a possible solution to improve productivity and reduce the cost of analysis within the toxicology laboratory. The ITSP system provides integrated online sample preparation which is controlled via the mass spectrometer software and utilizes disposable extraction cartridges.

The methods presented here for simultaneous quantification of many drugs demonstrate the power that can be achieved by coupling ITSP with LC/MS/MS. These methods have been validated according to FDA Bioanalytical Guidelines. They are in routine production in multiple clinical diagnostic laboratories. However, the validation and operation of a quantitative method of >50 compounds has many logistical challenges (e.g. preparation of standards) and operational cost (e.g. expense of labeled standards and longer chromatographic run times). Most labs have chosen to implement smaller focused tests (e.g. opiates) for faster sample analysis, along with the simplicity of ordering, data processing and billing.

Upon initial receipt by the SC Law Enforcement Division (SLED), urine samples from DUI-D cases were screened for amphetamine/methamphetamine, benzodiazepines, cocaine metabolite (benzoylecgonine), opiates, and THC metabolite (THCA) using Abbott Diagnostics fluorescence polarization immunoassay (FPIA). Previously validated confirmation methods using GC/MS or LC/MS/MS were utilized on samples which were positive on screening for one or more of the previously listed drug classes or had a history of drugs suspected. Additionally, samples that screened negative were further analyzed by a variety of GC/MS and LC/MS/MS methods if drug use was suspected (e.g. drugs found in the car). Aliquots of confirmed positive samples were supplied to OpAns for testing utilizing the ITSP/LC/MS/MS system. Confirmed positives covered all classes of drugs listed previously and accounted for over fifty different analytes of interest. All results provided in this study are from actual case samples.

Each sample submitted by SLED to OpAns for analysis by ITSP/ HPLC/ MS/MS was analyzed by two separate assays: one assay for THCA and barbiturates, the other assay for the remaining compounds of interest (>50 analytes). With the exception of glucuronide cleavage and centrifugation, each assay is fully automated and is performed in less than 10 minutes.



ITSP Design

Analytical syringe replaces standard column reservoir found in SPE and Filter media formats

Needle Penetrates Septum and Creates Seal So That When Plunger Is Depressed, Sample is Forced Through Media. Septum Also Grips Needle to Allow Instrument to Pick Up ITSP Cartridge for Movement.

Small Inner Diameter of ITSP Needle Guide Reduces Inner Volume While Assisting in Maintaining a Vertical Perpendicular Position

SPE or Sample Filtration Media

Sample Can Be Eluted into Collection Plate

Basic Extract Sample Preparation

ITSP SPE methods are very similar to other SPE methods with adjustments made for reduced sample and solvent volumes and the use of positive pressure. Samples to be analyzed for basic drugs were assembled for the PAL by combining 25 µL of internal standard, 25 µL of β-Glucuronidase in pH 4.5 buffer and 200 µL urine. The plates were sealed and allowed to incubate at 60ºC for 30 minutes with gentle mixing. The plate was centrifuged for 5 minutes at approximately 2000g.

1. Wash ITSP SPE cartridge with 100 µL of Solvent 1.
2. Condition ITSP SPE cartridge with 100 µL of water.
3. Load 200 µL of sample on the ITSP SPE cartridge.
4. Wash the ITSP cartridge with 100 µL of water.
5. Move ITSP cartridge over collection vial and Elute with 100 µL of Solvent 1.
Well contains 200 µL of 100 mM Ammonium Acetate in water.
6. Elute with 100 µL of Solvent 2 into the same vial.
7. Mix by aspirate/dispense.
8. Inject for LC/MS/MS analysis.
9. Peak areas were determined using Agilent MassHunter software.

Solvent 1 – 4:3:3:0.2 v/v THF:Methanol:Water:Ammonium Hydroxide
Solvent 2 – 5% Ammonium Hydroxide in water

212 Northlake Drive, Hartwell, GA 30643 • 855-395-8300 • www.ITSPsolutions.com

Apparatus

Autosampler:	CTC Analytics PAL System HPLC auto sampler or Gerstel MPS with ITSP hardware kit
HPLC:	Agilent Model 1200 SL with Binary Pump
MS:	Agilent Model 6430 QQQ

Analysis Conditions (Basic Analytes)

ITSP Cartridges: UCT SSDBX (MicroLiter 07-UDBX10-20A)

LC Conditions:

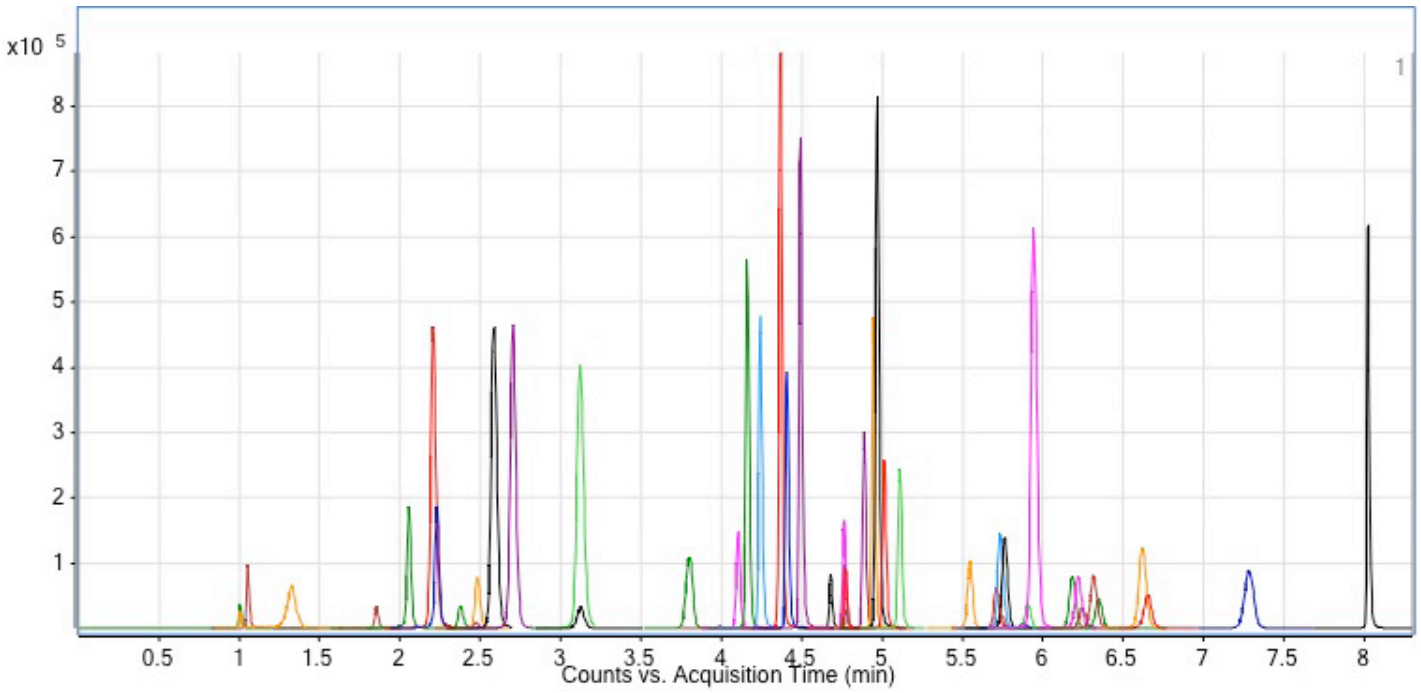
Solvent A:	Water with 0.1% (v/v) Formic Acid
Solvent B:	Methanol with 0.1% (v/v) Formic Acid
Column:	50 x 3mm i.d., Poroshell 120 EC-C18, 2.7 µm (Agilent)
Injection Vol.:	10 µL
Column Temperature:	30ºC
Flowrate:	0.8 mL/min

Gradient:

Time (min)	0.0	0.10	1.00	3.00	4.20	7.00	7.75	8.25	8.30
%B	3	3	15	20	50	55	100	100	3

MS Conditions:

Instrument:	Agilent 6430 Triple Quadrupole
Ionization Mode:	Electrospray @ 350ºC
Polarity:	Positive
Transitions:	Available upon request



Analytes of Interest Include:

RT	Compound	RT	Compound	RT	Compound
0.97	Morphine	3.73	Benzoylecgonine	5.09	Buprenorphine
1.00	Noroxycodone	3.96	Norfentanyl	5.55	Nitrazepam
1.00	Oxymorphone	4.12	7-Amino Flunitrazepam	5.68	Propoxyphene
1.25	Hydromorphone	4.14	Tramadol	5.69	Clonazepam
1.27	Norcodeine	4.19	Cocain	5.72	a-OH Triazolam
1.83	Dihydrocodeine	4.22	Methylphenidate	5.74	Flunitrazepam
1.84	Codeine	4.35	Tapentadol	5.81	Norpropoxyphene
1.86	Norhydrocodone	4.39	Meperidine	5.87	Methadone
2.03	Oxycodone	4.47	Normeperidine	5.89	a-OH Alprazolam
2.15	Amphetamine	4.66	PCP	6.15	Carisoprodol
2.20	Hydrocodone	4.75	Fentenyl	6.20	Alprazolam
2.31	Methamphetamine	4.75	Norbuprenorphine	6.29	Oxazepam
2.41	MDA	4.76	Meprobamate	6.31	Lorazepam
2.43	6-MAM	4.79	Chlordiazepoxide	6.58	Temazepam
2.52	MDMA	4.88	Midazolam	6.73	Nordiazeapam
2.67	O-Desmethyltramadol	4.92	EDDP	7.33	Diazepam
3.04	MDEA	4.95	Flurazepam	8.03	Prazepam
3.10	7-Amino Clonazepam	5.05	a-OH Midazolam		

212 Northlake Drive, Hartwell, GA 30643 • 855-395-8300 • www.ITSPsolutions.com

Analysis Conditions (Acidic Analytes)

ITSP Cartridges: UCT CSDAU 10 mg (MicroLiter 07-UDAU10-20A)

LC Conditions:

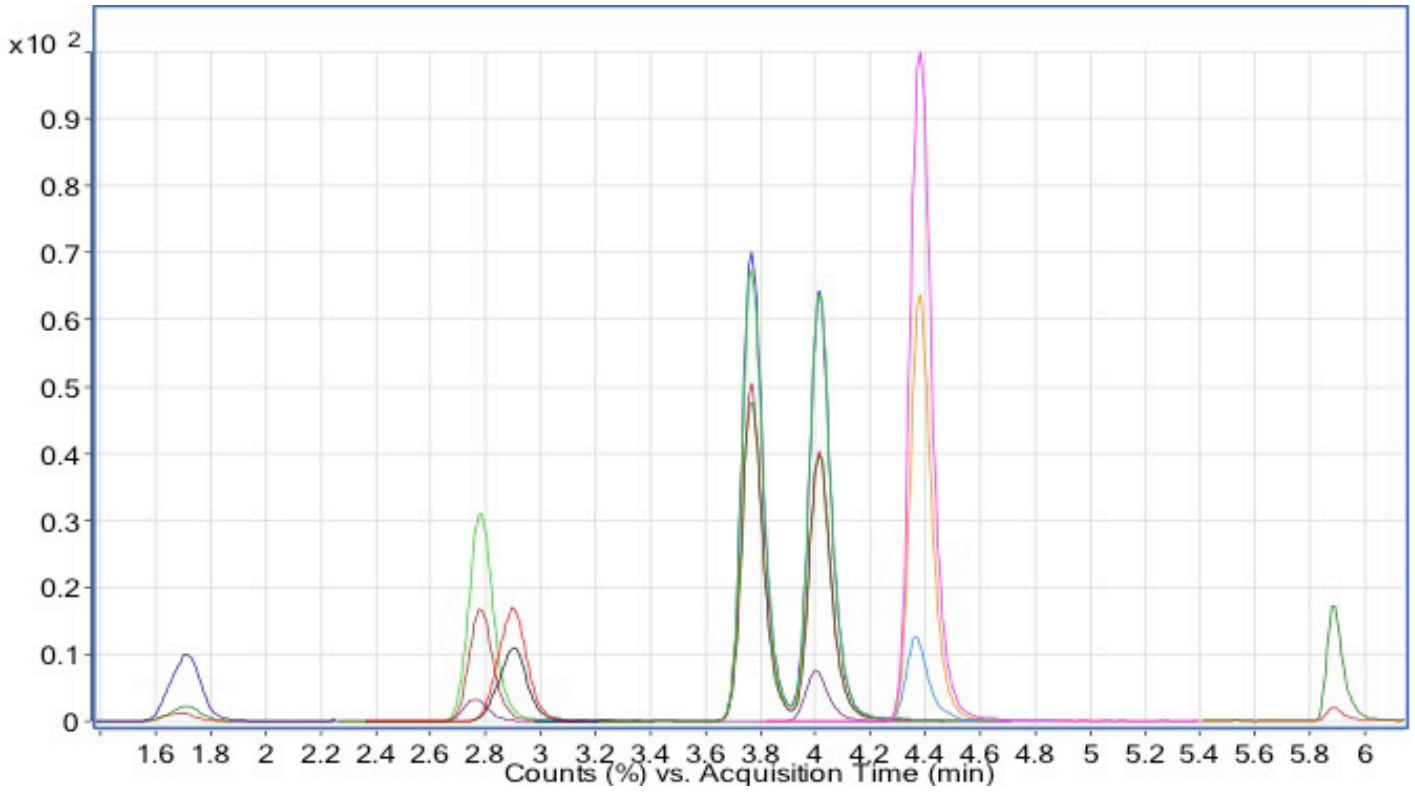
Solvent A:	Water with 5 mM ammonium acetate and 0.05% (v/v) Ammonium Hydroxide
Solvent B:	Methanol with 0.05% Ammonium Hydroxide
Column:	50 x 2.1 mm i.d., XTerra MS C18, 3.5 µm (Waters)
Injection Vol.:	10 µL
Column Temperature:	30ºC
Flowrate:	0.5 mL/min

Gradient:

Time (min)	0.0	0.50	4.00	6.50	7.00	7.50
%B	5	5	45	100	100	5

MS Conditions:

Instrument:	Agilent 6430 Triple Quadrupole
Ionization Mode:	Electrospray @ 350ºC
Polarity:	Negative
Transitions:	Available upon request



Analytes of Interest Include:

RT	Compound
1.7	Phenobarbital
2.7	Butalbital
2.8	Butabarbital
3.7	Amobarbital
3.9	Pentobarbital
4.3	Secobarbital
5.9	11-Carboxy-THC

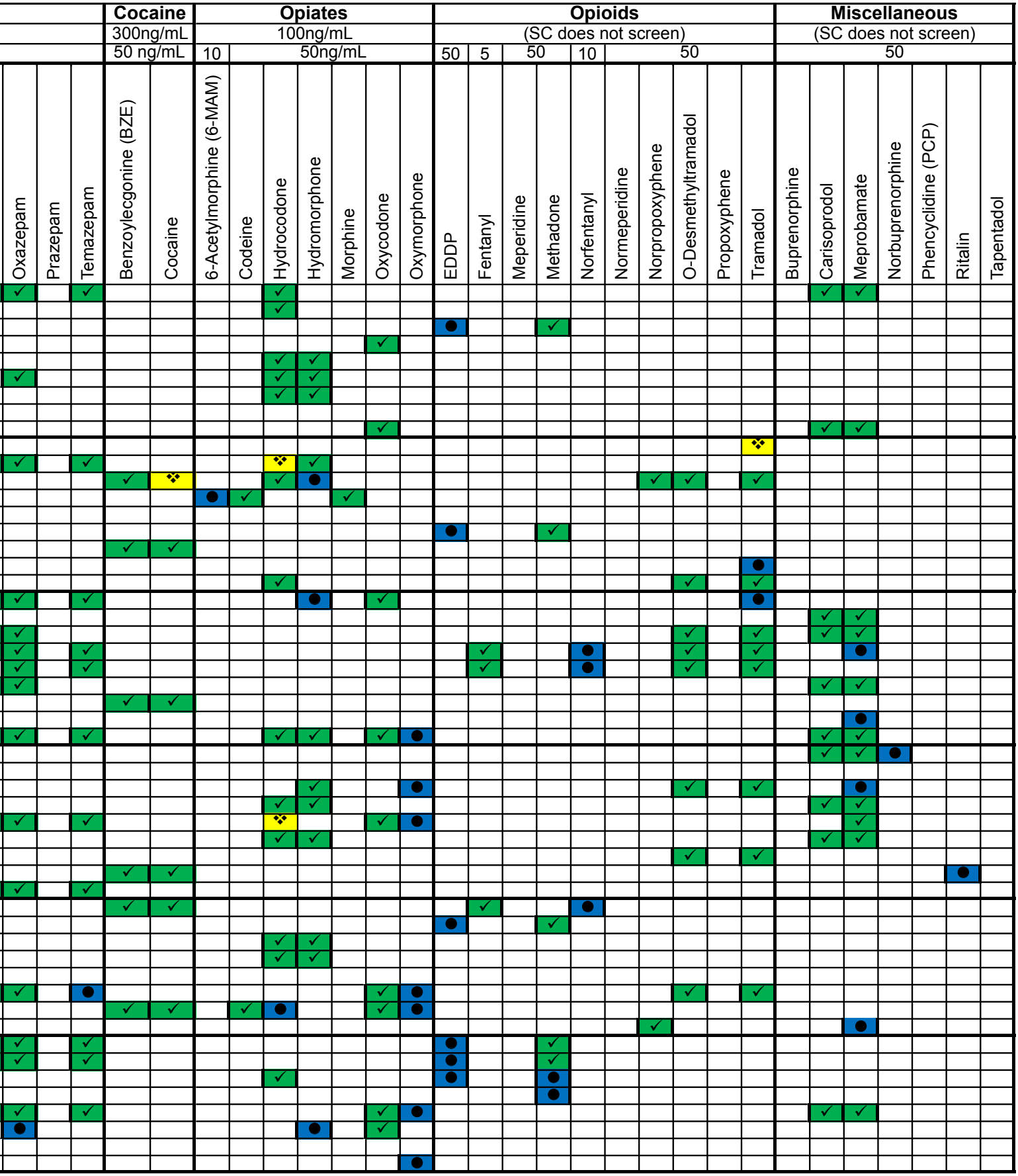
	Barbiturates						THC	Amphetamines					Benzodiazepines																
SC	(SC does not screen)						100	1000ng/mL					10ng/mL																
Op	50ng/mL						10	50ng/mL					50ng/mL																
Sample	Amobarbital	Butabarbital	Butalbital	Pentobarbital	Phenobarbital	Secobarbital	THCA	Amphetamine	MDA	MDEA	MDMA	Methamphetamine	2-Hydroxyethyl Flurazepam	7-Amino Clonazepam	7-Amino Flunitrazepam	Alpha-Hydroxy Midazalam	Alpha-Hydroxy Triazolam	Alpha-Hydroxyalprazolam	Alprazolam	Chlordiazepoxide	Clonazepam	Diazepam	Flunitrazepam	Flurazepam	Lorazepam	Midozalam	Nitrazepam	Nordiazepam
1																						✓							✓
2							✓																						
3																		✓	✓										
4																									✓				
5																													
6														✓				✓	✓						✓				
7																													
8							✓							✓				✓	✓										
9							✓																		●				
10																		✓	✓										
11		✓						●										✓	✓			✓							✓
12																		✓	✓										
13																													
14							✓							✓				✓	✓		✓								
15																		✓	✓										
16							✓																						
17																									✓				
18								✓																	●				
19					✓									✓							✓	✓							✓
20							●	✓																					
21																		✓	●										✓
22																						✓							✓
23																						✓							
24																		✓	✓										✓
25																													
26																													
27							✓											✓	✓										✓
28								●																					
29							●											✓	✓										
30																													
31																		✓	✓										
32																						✓							✓
33														✓				✓	✓		✓								
34							✓							●															
35								✓																					
36																		✓	✓			✓							✓
37														●				✓	✓										
38							✓											✓	✓						✓				
39																		✓	✓										
40							✓																						
41																		✓	✓						✓				
42																			✓										✓
43							●							✓				✓	✓										
44							●											✓	✓										
45																						✓							✓
46								✓						✓				✓	✓			✓							✓
47																													
48							✓		✓		✓																		
49							●																						✓
50							●																						
51							✓											✓	✓										
52							✓											✓	✓										

Totals: 470 32

Not Detected by both SC and OpAns

✓ Confirmed by both SC and OpAns

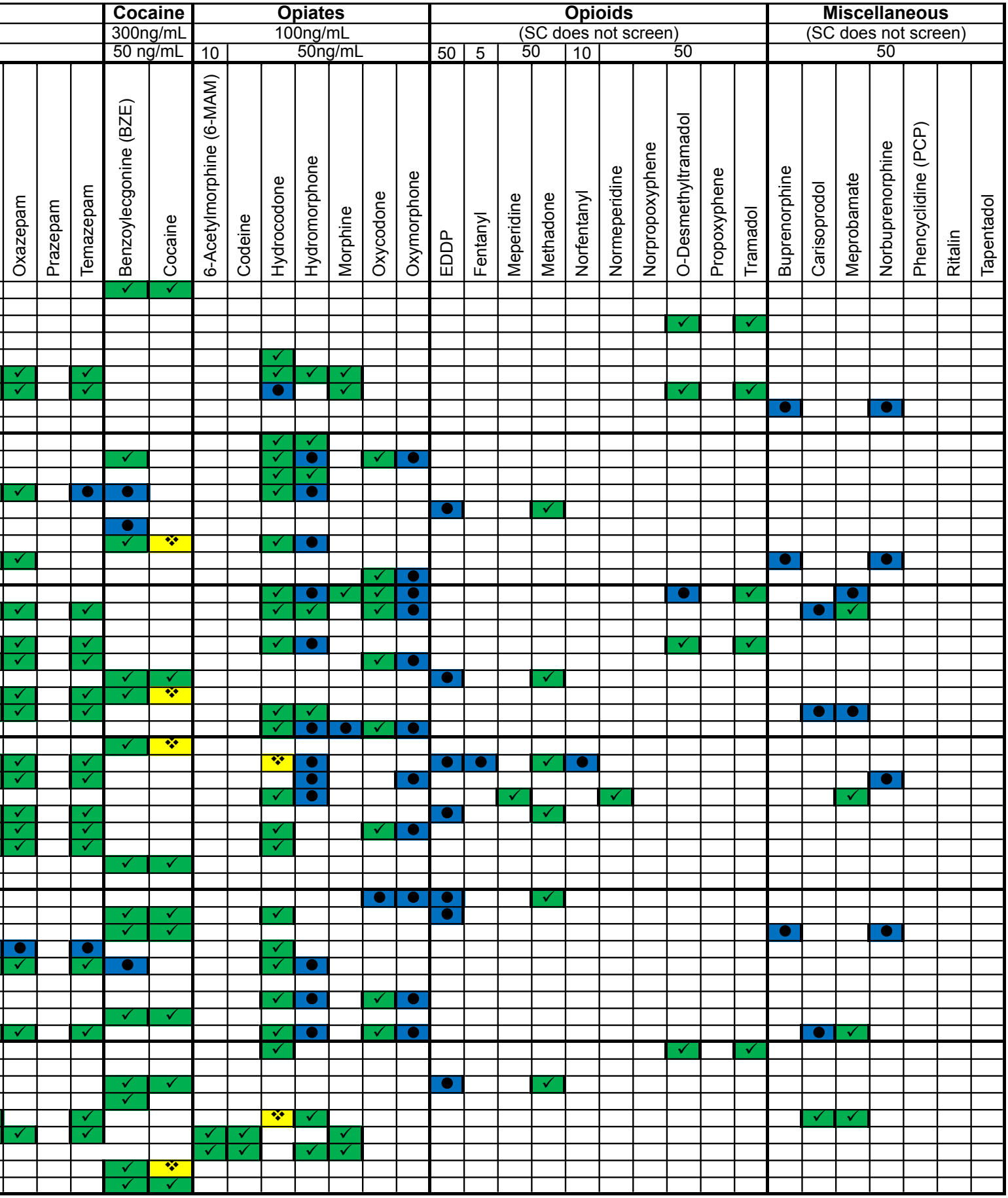
❖ SC Confirmed & OpAns Detected


Cocaine
300ng/mL
50 ng/mL
Opiates
100ng/mL
10
50ng/mL
Opioids
(SC does not screen)
50
5
50
10
50
Miscellaneous
(SC does not screen)
50
Oxazepam
Prazepam
Temazepam
Benzoylecgonine (BZE)
Cocaine
6-Acetylmorphine (6-MAM)
Codeine
Hydrocodone
Hydromorphone
Morphine
Oxycodone
Oxymorphone
EDDP
Fentanyl
Meperidine
Methadone
Norfentanyl
Normeperidine
Norpropoxyphene
O-Desmethyltramadol
Propoxyphene
Tramadol
Buprenorphine
Carisoprodol
Meprobamate
Norbuprenorphine
Phencyclidine (PCP)
Ritalin
Tapentadol
0
X
Confirmed by SC but not by OpAns
95
OpAns Confirmed and SC Not Reported

	Barbiturates						THC	Amphetamines					Benzodiazepines																
SC	(SC does not screen)						100	1000ng/mL					10ng/mL																
Op	50ng/mL						10	50ng/mL					50ng/mL																
Sample	Amobarbital	Butabarbital	Butalbital	Pentobarbital	Phenobarbital	Secobarbital	THCA	Amphetamine	MDA	MDEA	MDMA	Methamphetamine	2-Hydroxyethyl Flurazepam	7-Amino Clonazepam	7-Amino Flunitrazepam	Alpha-Hydroxy Midazalam	Alpha-Hydroxy Triazolam	Alpha-Hydroxyalprazolam	Alprazolam	Chlordiazepoxide	Clonazepam	Diazepam	Flunitrazepam	Flurazepam	Lorazepam	Midozalam	Nitrazepam	Nordiazepam
53																													
54							✓											✓	✓										
55																		✓	✓						✓				
56																									✓				
57														✓							✓								
58																						✓						✓	
59			✓																									✓	
60																		✓	✓										
61																		✓	✓						✓				
62														✓				✓	✓		✓								
63																		✓	✓										
64							●	✓																					
65							✓	✓				✓		✓				✓	✓									✓	
66							✓	✓				✓		✓				✓	●										
67							✓							✓				✓	✓										
68							✓	✓				✓																	
69																				✓								✓	
70							✓																						
71							●	✓				✓																	
72														✓				✓	✓		✓	✓						✓	
73								✓										✓	✓										
74																		✓	✓			✓							
75							✓	●														✓						✓	
76							✓											✓	✓										
77							●							✓														✓	
78																												✓	
79							●							●															
80							✓																						
81																		✓	✓									✓	
82								✓																				✓	
83																		✓	✓										
84								✓																				✓	
85																												✓	
86							●	✓				✓		●				✓	✓			✓						✓	
87												●													●				
88							✓	✓				✓						✓	✓										
89							✓											✓	✓										
90							✓																						
91							✓	✓						✓				✓	✓										
92																		✓	✓									●	
93							●																					✓	
94							✓											✓	✓										
95							✓											✓	✓										
96																													
97																						✓						✓	
98			✓															✓	✓										
99																		✓	✓										
100																		✓	✓										
101							✓																						
102							✓											✓	✓			✓						✓	
103																									✓			✓	
104							✓																						
105																													
106																													

Totals:

	Not Detected by both SC and OpAns	470 ✓	Confirmed by both SC and OpAns
		32 ❖	SC Confirmed & OpAns Detected


Cocaine
300ng/mL
50 ng/mL
Opiates
100ng/mL
10
50ng/mL
Opioids
(SC does not screen)
50
5
50
10
50
Miscellaneous
(SC does not screen)
50
Oxazepam
Prazepam
Temazepam
Benzoylecgonine (BZE)
Cocaine
6-Acetylmorphine (6-MAM)
Codeine
Hydrocodone
Hydromorphone
Morphine
Oxycodone
Oxymorphone
EDDP
Fentanyl
Meperidine
Methadone
Norfentanyl
Normeperidine
Norpropoxyphene
O-Desmethyltramadol
Propoxyphene
Tramadol
Buprenorphine
Carisoprodol
Meprobamate
Norbuprenorphine
Phencyclidine (PCP)
Ritalin
Tapentadol
0
X
Confirmed by SC but not by OpAns
95
OpAns Confirmed and SC Not Reported

Discussion

The ITSP methods used in this poster were originally developed for application to the clinical field of pain management. These methods have proven to be sufficiently robust to process forensic urine samples without modification. One hundred six (106) samples were submitted for testing using ITSP coupled to LC/MS/MS.

Review of the data summary reveals the following:

Barbiturates: SLED only tests for barbiturates when suspected. All samples previously confirmed by SLED as containing barbiturates were confirmed by OpAns.

THC metabolite: Twelve additional cases were found to contain THC-COOH when evaluated by OpAns. All twelve samples screened negative by FPIA at the established cut off of 100 ng/mL. The additional positive cases can be attributed to the lower ITSP Limit of Quantification of 10 ng/mL for THC-COOH.

Amphetamines: Four additional samples were found to contain amphetamine or methamphetamine when evaluated by OpAns. All four samples screened negative by FPIA at the established cut off of 1000 ng/mL. The additional positive cases can be attributed to the lower ITSP Limit of Quantification of 50 ng/mL.

Benzodiazepines: Six additional samples were found to contain one or more benzodiazepines when evaluated by OpAns. All six samples screened negative by FPIA at the established cut off of 200 ng/mL. Additional benzodiazepines were confirmed by OpAns in nine cases.

Cocaine/cocaine metabolite: All samples previously confirmed by SLED as containing cocaine or benzoylecgonine were found to contain cocaine and benzoylecgonine upon analysis by OpAns. In a few instances, the concentration of cocaine had decreased so that it was less than the OpAns 50ng/mL cut-off. Three additional samples were found to contain benzoylecgonine when evaluated by OpAns. All three samples screened negative by FPIA at the established cut off of 300 ng/mL.

Opiates: Three additional samples were found to contain one or more opiates when evaluated by OpAns. All three samples screened negative by FPIA at the established cut off of 100 ng/mL. Oxymorphone was confirmed in a total of 17 cases. Oxymorphone is not currently a target analyte of SLED's normal opiate panel. Sample 13 was found to contain 6-monoacetylmorphine when evaluated by OpAns. This sample was originally reported to contain codeine and morphine. The detection of 6-monoacetylmorphine by OpAns may be attributed to the 10 ng/mL LOQ established for the ITSP method.

Opioids/Miscellaneous: Currently, SLED does not perform routine screens for any of the drugs listed in these categories. General acid, base, neutral extractions followed by GC/MS and LC/MS/MS analysis are preformed if a history of suspected drugs is provided and normal presumptive screens are negative.

All differences in results between the methods can be explained.

Conclusions

More drugs were found using simultaneous screening/confirmation by ITSP/HPLC/MS/MS than traditional immunoassay screening with single drug class confirmation.

One operator can process 50 case samples per day through both methods on one ITSP/HPLC/MS/MS.

Current costs of expendable supplies for a five panel drug screen (FPIA) and a single confirmation utilizing traditional SPE and GC/MS or LC-MS/MS average $16.50. Supplies for additional confirmations average $7.00.

The total for all supplies to perform both ITSP/HPLC/MS/MS methods is $12 per sample.

Analysis using ITSP/HPLC/MS/MS produces comprehensive results in less time for less money than conventional screening with immunoassay followed by GC/MS and/or LC/MS/MS confirmation.

Acknowledgments

Special thanks to Thurman Allsup for analytical support and analysis of urine specimens by ITSP.

For Further Information Contact:

Ken Lewis at KLewis@OpAns.com (919) 323-4299
Kim Gamble at Kim.Gamble@ITSPsolutions.com (855) 395-8300

ITSP Automation Development Questionnaire

Tell us about yourself and your interest in ITSP:

I am Mr., Mrs., Miss _____

I work for _____

My position is _____

My Address is _____

_____

_____

City _____ State _____ Zip _____

Country _____

Telephone number _____

Email address _____

Fax number _____

Are you in charge of or have the ability to authorize the use of ITSP if we work with you to demonstrate ITSP in your lab?

_____

It is okay if you are not but we will need to know who has that authority and whether or not they are open to discussions regarding our technology. My superior who will need to be involved is:

Mr., Mrs., Miss _____ .

Telephone number _____

Please describe why you believe ITSP can benefit your laboratory:

_____

_____

_____

_____

_____

When would be a good time to call you about your application? 1st choice _____ 2nd choice _____

ITSP SPE Automation Development Questionnaire

Tell us about your instrument.

From this information we may suggest additional hardware, please complete one questionnaire for each application and instrument.

I have a CTC Analytics Combi PAL_____, Combi Headspace PAL_____, HTS PAL_____, HTC PAL _____.
The company who sold me my PAL is _____.
My sales rep from this company is _____ phone no. (___) _____
I do not have a PAL. My application is for GC-based _____ or LC-based _____ chromatography.

I have the following accessories for my HTS or HTC PAL:

_____ Fast Wash Station
_____ 3 or 6 Drawer Stack
_____ 2 Position Microplate Holder
_____ 12x32mm Vial Tray
_____ Other (please specify) _____
_____ 3-Position Ink-Well Solvent Reservoir
_____ 3 Drawer Cool Stack
_____ 4 Position Microplate Holder
_____ Syringe Adapter, _____ Volume

I have the following accessories for my Combi PAL or Combi Headspace PAL

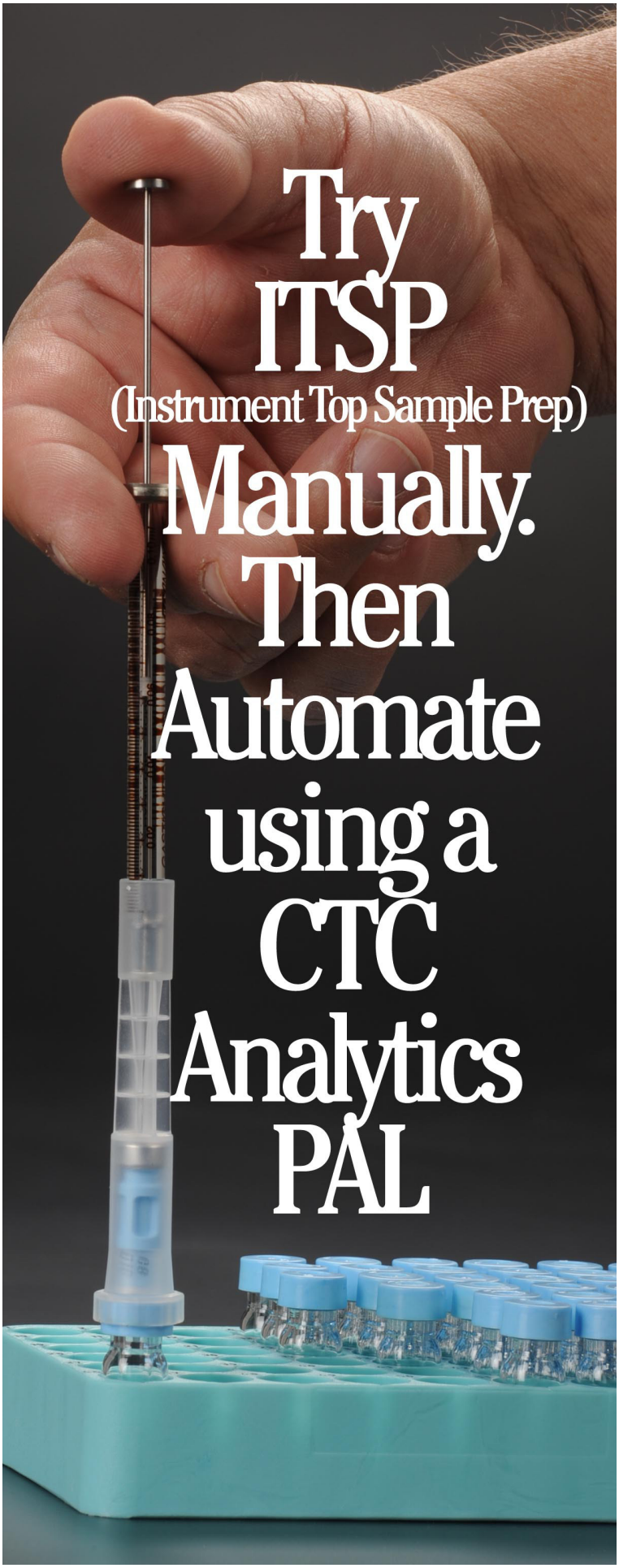
_____ Agitator
_____ Syringe Adaptor, _____ Volume
_____ 3 Drawer Stack
_____ Other (please specify) _____
_____ 5 position 10mL Solvent Reservoir
_____ 12x32mm Vial Tray
_____ 3 Drawer Cool Stack

Tell us about your method:

I am starting from scratch but want to extract _____ from _____ .
I do not have a developed method.
I am extracting or want to extract _____from _____.
Sorbent _____ Manufacturer _____
Bedmass (mg) _____
1st Conditioning Solvent Volume _____ 2nd Conditioning Solvent Volume _____
Raw Sample Volume _____ Contained in what type of container _____diameter (mm) _____
Sample Load Volume _____
1st Column Wash Volume _____ 2nd Column Wash Volume (if applicable) _____
3rd Column Wash Volume _____ 4th Column Wash Volume _____
Do you need to dry the sorbent after column wash? _____
Elution Volume _____
Do you need to dry down and reconstitute the eluted sample? _____
Volume of solvent for reconstitution? _____
Injection Volume _____
Samples prepped per day _____ month _____ year _____
My analytical instrument's sample analysis time is _____ minutes _____ seconds.

How many ITSP devices do you need to manually try your method _____? (Provided FREE of charge)
ITSP Solutions will take the Proof-of-Concept method you develop using our device and offer you an off-line demonstration using our PAL or work with your local PAL sales rep if you do not have a PAL. However please understand that they have no obligation to assist us and to do so may incur additional expense.

Do you need assistance with Method Development or scaling down for automation using ITSP? _____ .
While ITSP Solutions has a relationship with OpAns, Inc. to develop applications using ITSP, we must charge for services related to method development, optimization and validation. It may also be important to involve the installer of the PAL system so that we integrate our system without impacting the system as if currently exists.



ITSP™
SOLUTIONS, INC.

212 Northlake Drive, Hartwell, GA 30643 • 855-395-8300
www.ITSPsolutions.com • 706-395-8300