



# **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<sup>1</sup>, Kenneth Lewis, CEO<sup>2</sup>, and Kim Gamble, President<sup>3</sup>

<sup>1</sup>SC Law Enforcement Division, Columbia, SC 29221

<sup>2</sup>OpAns LLC, Durham, NC 27713, <sup>3</sup>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.

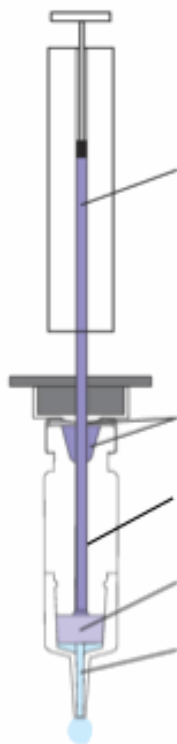
# Background

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

ITSP is protected under the following US PTO Patents: 6,859,615, 7,001,774 & 7,798,021. European Patents 1 74 701 and 1 808 700. Canadian Patents 2,316,648. Other patents pending.

## 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  $\mu\text{L}$  of internal standard, 25  $\mu\text{L}$  of  $\beta$ -Glucuronidase in pH 4.5 buffer and 200  $\mu\text{L}$  urine. The plates were sealed and allowed to incubate at 60°C for 30 minutes with gentle mixing. The plate was centrifuged for 5 minutes at approximately 2000g.

1. Wash ITSP SPE cartridge with 100  $\mu\text{L}$  of Solvent 1.
2. Condition ITSP SPE cartridge with 100  $\mu\text{L}$  of water.
3. Load 200  $\mu\text{L}$  of sample on the ITSP SPE cartridge.
4. Wash the ITSP cartridge with 100  $\mu\text{L}$  of water.
5. Move ITSP cartridge over collection vial and Elute with 100  $\mu\text{L}$  of Solvent 1.  
Well contains 200  $\mu\text{L}$  of 100 mM Ammonium Acetate in water.
6. Elute with 100  $\mu\text{L}$  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

## 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:

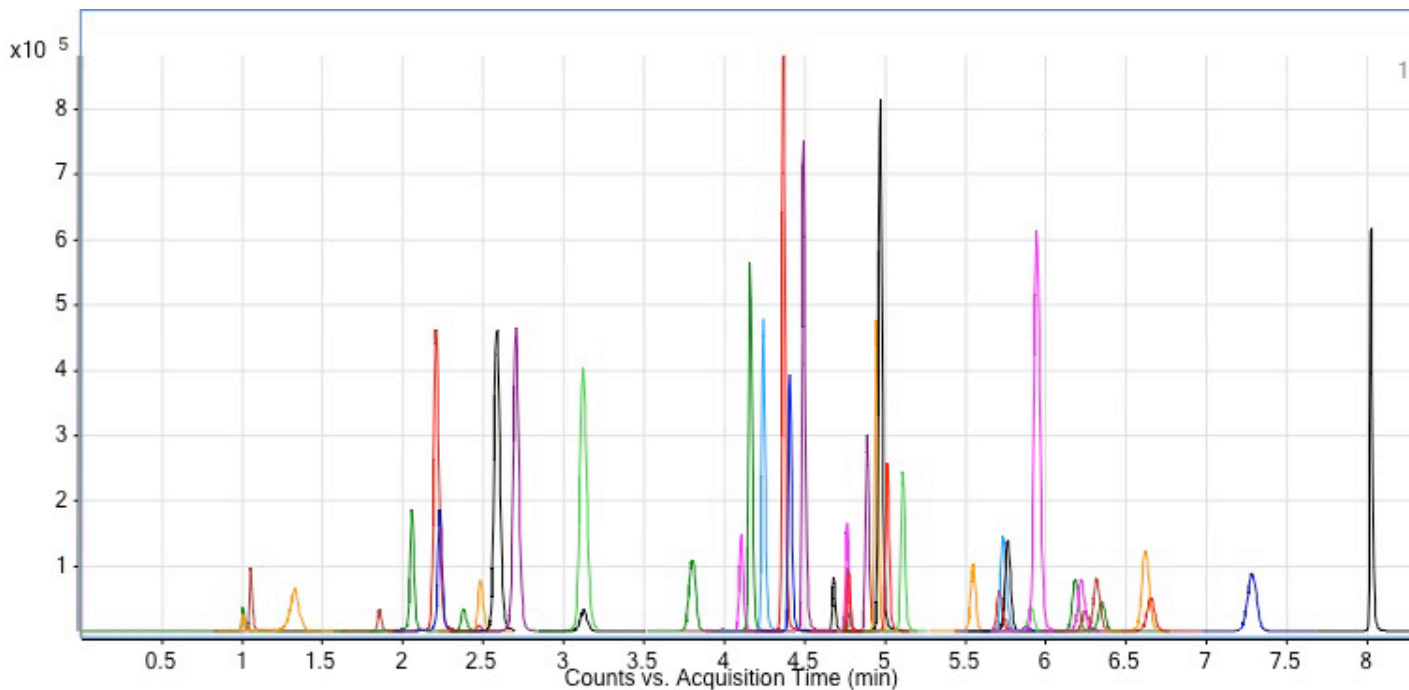
Solvent A: Water with 0.1% (v/v) Formic Acid  
Solvent B: Methanol with 0.1% (v/v) Formic Acid  
Column: 50 x 3mm i.d., Poroshell 120 EC-C18, 2.7  $\mu$ m (Agilent)  
Injection Vol.: 10  $\mu$ L  
Column Temperature: 30°C  
Flowrate: 0.8 mL/min

### Gradient:

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

### MS Conditions:

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



## Analytes of Interest Include:

<b>RT Compound</b>	<b>RT Compound</b>	<b>RT Compound</b>
0.97 Morphine	3.73 Benzoylcegonine	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 Fentanyl	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 Nordiazepam
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	

## Analysis Conditions (Acidic Analytes)

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

### **LC Conditions:**

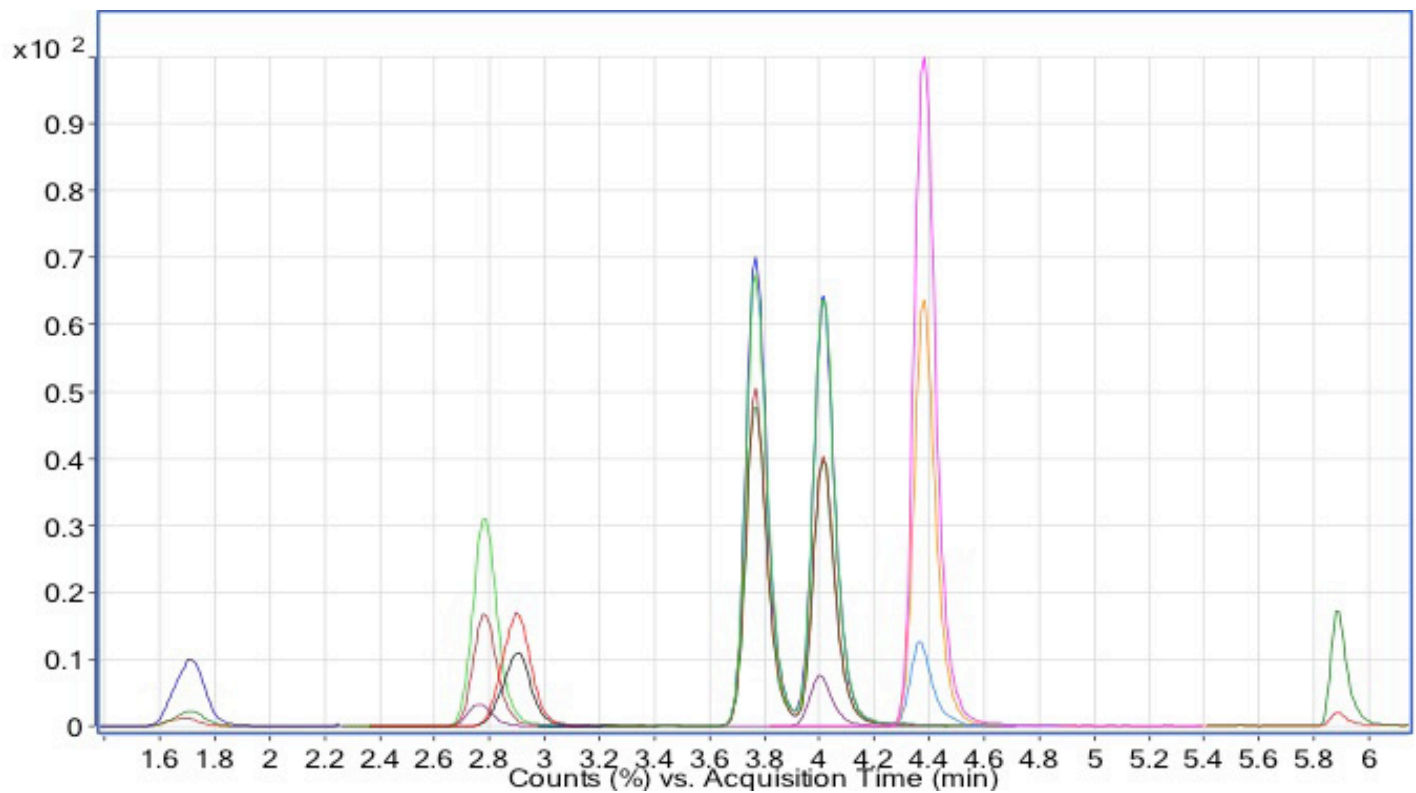
**Solvent A:** Water with 5 mM ammonium acetate and 0.05% (v/v) Ammonium Hydroxide  
**Solvent B:** Methanol with 0.05% Ammonium Hydroxide  
**Column:** 50 x 2.1 mm i.d., XTerra MS C18, 3.5 µm (Waters)  
**Injection Vol.:** 10 µL  
**Column Temperature:** 30°C  
**Flowrate:** 0.5 mL/min

### **Gradient:**

<b>Time (min)</b>	0.0	0.50	4.00	6.50	7.00	7.50
<b>%B</b>	5	5	45	100	100	5

### **MS Conditions:**

**Instrument:** Agilent 6430 Triple Quadrupole  
**Ionization Mode:** Electrospray @ 350°C  
**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

SC Op	Barbiturates						THC	Amphetamines					Benzodiazepines																
	(SC does not screen)						100	1000ng/mL					10ng/mL																
	50ng/mL						10	50ng/mL					50ng/mL																
Sample	Amobarbital	Butabarbital	Butalbital	Pentobarbital	Phenobarbital	Secobarbital	THCA	Amphetamine	MDA	MDEA	MDMA	Methamphetamine	2-Hydroxyethyl Fluorazepam	7-Amino Clonazepam	7-Amino Flunitrazepam	Alpha-Hydroxy Midazolam	Alpha-Hydroxy Triazolam	Alpha-Hydroxyalprazolam	Alprazolam	Chlordiazepoxide	Clonazepam	Diazepam	Flunitrazepam	Flurazepam	Lorazepam	Midazolam	Nitrazepam	Nordiazepam	
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Totals:

Not Detected by both SC and OpAns

470

Confirmed by both SC and OpAns

32

SC Confirmed & OpAns Detected



			Cocaine		Opiates						Opioids						Miscellaneous												
			300ng/mL		100ng/mL						(SC does not screen)						(SC does not screen)												
			50 ng/mL		10	50ng/mL					50	5	50		10	50			50										
Oxazepam	Prazepam	Temazepam	Benzoylcegonine (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	
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Confirmed by SC but not by OpAns

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OpAns Confirmed and SC Not Reported

Sample	Barbiturates					THC	Amphetamines				Benzodiazepines																	
	(SC does not screen)					100	1000ng/mL				10ng/mL																	
	50ng/mL					10	50ng/mL				50ng/mL																	
	Amobarbital	Butabarbital	Butalbital	Pentobarbital	Phenobarbital	Secobarbital	THCA	Amphetamine	MDA	MDEA	MDMA	Methamphetamine	2-Hydroxyethyl Flurazepam	7-Amino Clonazepam	7-Amino Flunitrazepam	Alpha-Hydroxy Midazolam	Alpha-Hydroxy Triazolam	Alpha-Hydroxyalprazolam	Alprazolam	Chlordiazepoxide	Clonazepam	Diazepam	Flunitrazepam	Flurazepam	Lorazepam	Midazolam	Nitrazepam	Nordiazepam
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Totals:

Not Detected by both SC and OpAns

470  Confirmed by both SC and OpAns

32  SC Confirmed & OpAns Detected

			Cocaine		Opiates						Opioids						Miscellaneous												
			300ng/mL	50 ng/mL	100ng/mL						(SC does not screen)						(SC does not screen)												
				10	50ng/mL						50	5	50		10	50			50										
Oxazepam	Prazepam	Temazepam	Benzylecgonine (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	
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# 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 performed 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

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### **For Further Information Contact:**

Ken Lewis at [KLewis@OpAns.com](mailto:KLewis@OpAns.com) (919) 323-4299

Kim Gamble at [Kim.Gamble@ITSPsolutions.com](mailto: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-Position Ink-Well Solvent Reservoir
_____ 3 or 6 Drawer Stack	_____ 3 Drawer Cool Stack
_____ 2 Position Microplate Holder	_____ 4 Position Microplate Holder
_____ 12x32mm Vial Tray	_____ Syringe Adapter, _____ Volume
_____ Other (please specify) _____	

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

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

## 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 \_\_\_\_\_

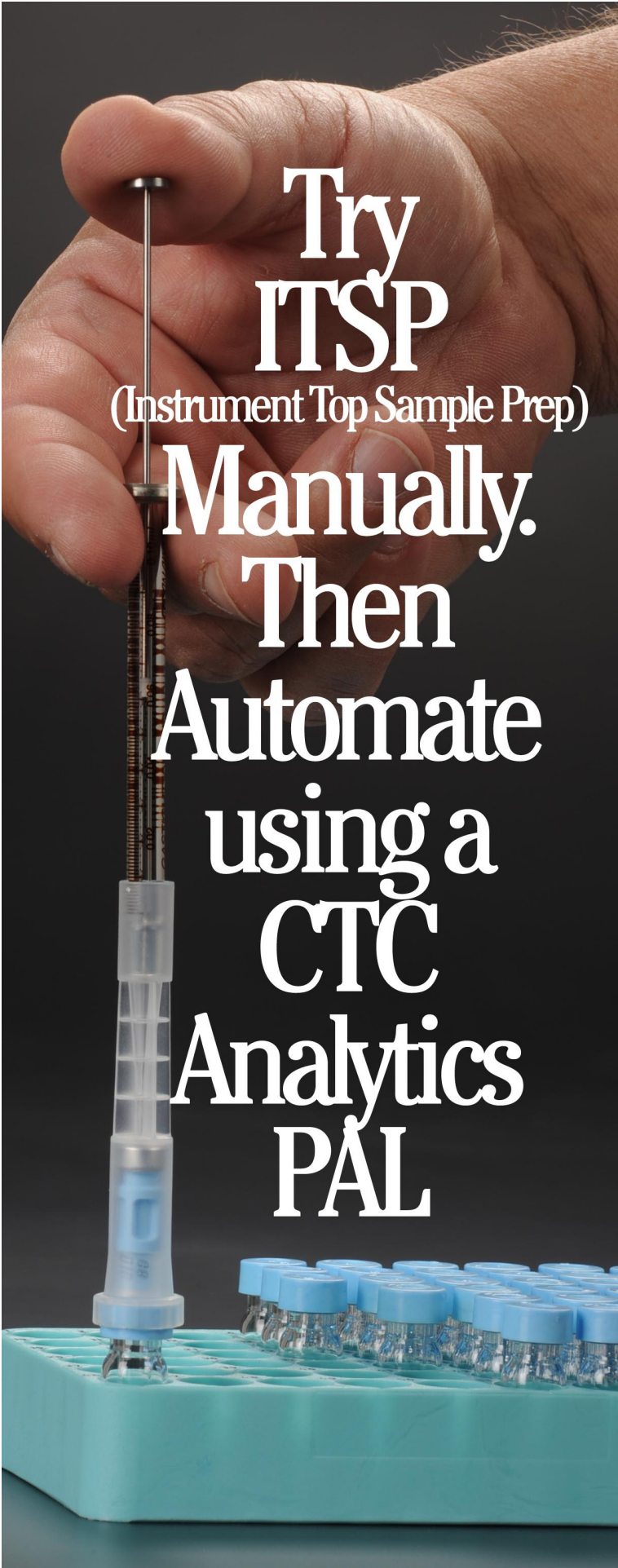
My analytical instrument's sample analysis time is \_\_\_\_\_ minutes \_\_\_\_\_ seconds.

## 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.



Try  
ITSP  
(Instrument Top Sample Prep)  
Manually.  
Then  
Automate  
using a  
CTC  
Analytics  
PAL



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