ITSP® SOLUTIONS, INC.

Instrument Top Sample Prep

A consumable solution for automation and the best measurement performance!

Pain Management & Drugs of Abuse Measured in Urine and/or Oral Fluid

Measuring all the drugs at all the relevant concentrations in a single unified method / workflow using an ITSP solution for on-line SPE-LC/MS/MS

Developed in collaboration with Assurance Scientific Laboratories

71+ Drugs, 4.5 Minutes Inject-to-Inject,
Two 96-position trays per instrument overnight

Introduction

The measurement of drugs of abuse in urine and/or oral fluid is common for pre-employment screening and DOT / federally mandated testing. However, the most rapid expansion of testing has been in the fields of law enforcement and compliance monitoring / diagnostic evaluation by physicians. Although many methods are available for these measurements, the fastest growing and preferred approach is liquid chromatography tandem mass spectrometry (LC/MS/MS) because of the high degree of certainty it affords determining concentration and identification of compounds. While the continued growth of the use of LC/MS/MS for the measurement of drugs of abuse in urine and oral fluid seems certain, there are still several technical challenges that need to be met. These needs include being able to easily measure low-dose drugs at or near 1ng/g concentration (for medical purposes, Pesce, et. al. 2012 AACC conference and zero tolerance testing), simplicity for performing measurements by lab technicians with relatively little training, and the ability to achieve high throughput for all work while minimizing the labor and number of workflows required.

In an effort to meet these needs, Instrument Top Sample Prep Solutions, Inc. (ITSP) along with Assurance Scientific Laboratories has developed the automated on-line ITSP-LC/MS/MS method described here. This ITSP solution uses on-line Solid Phase Extraction (SPE) to clean and pre-concentrate urine or oral fluid (using the Quantisal OF sample collection device) samples so that low-dose drugs at or near 1ng/g concentration are easily measured at S/N ≥20. At the same time, the method's design is balanced to identify and measure all of the drugs (acidic and basic drugs as well as polar and non-polar drugs in urine or oral fluid) in one ITSP SPE method, all in one unified LC/MS/MS workflow. It is simple, robust, and can be performed by lab technicians with MS familiarity. It is completely automated from sample plates or vials to results (using the native MS software) and can process two 96-well plates of samples overnight per LC/MS/MS. The results will be waiting for you in the morning.

Total automation is achieved using the PAL System LC sample handler (the most commonly used autosampler for GC/MS/MS and LC/MS/MS worldwide). Since one must invest in an autosampler as a prerequisite to LC/MS/MS, we recommend choosing one that can prepare the sample as well as inject it. The cycle time achieved for on-line ITSP-LC/MS/MS is 4.5 minutes for 71 drugs in urine (opiates, metabolites, illicits, opioids, barbs, benzos, and THCA) and a representative 4.1 minute chromatogram is shown below.

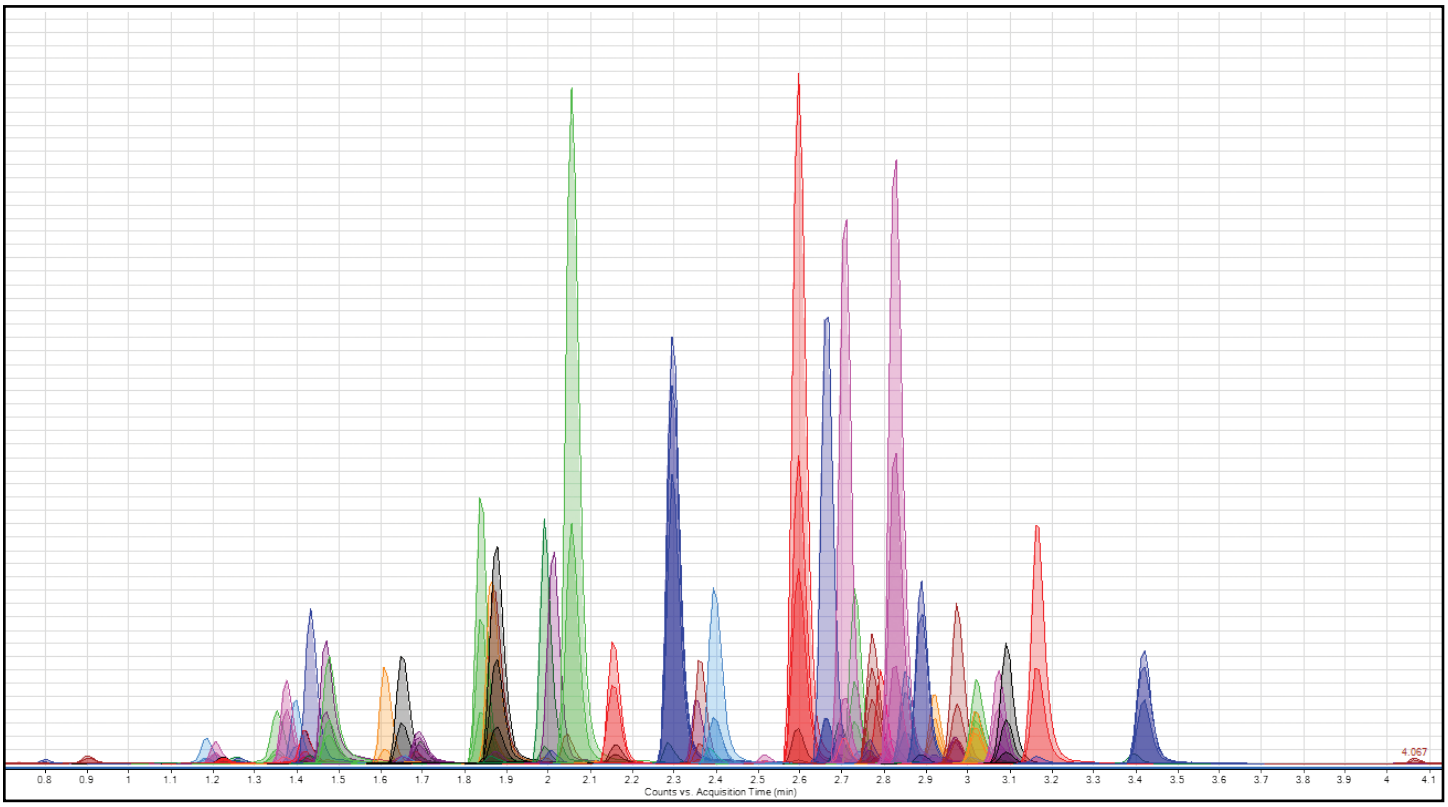


Figure 1: Total automation of the ITSP Solid Phase Extraction then analysis by LC/MS/MS (ITSP-LC/MS/MS) is routinely performed in the clinical production environment for 71 drugs (basic and acidic) in one injection in 4.5 minutes, injection to injection. Additional compounds can be added or removed based on the individual needs of the lab.

Sample Preparation - ITSP Reverse Phase SPE Conditions

Sample preparation

Oral fluid samples require either centrifugation or filtering (Quantisal filter) and on occasion, urine samples may require this too. All standards and their isotopically labeled internal standards may be obtained from Cerilliant. Standards of the drugs should be prepared in urine (or oral fluid) at appropriate concentrations across the analytical range. Isotopically labeled internal standards also should be added to the sample. ß-Glucuronidase (5000 Fishman Units) in 1M pH 4.5 buffer should be added to each urine sample (not oral fluid) and heated at 60°C for 3 hours using these steps:

Step-by-Step Process

1) To each well or vial (except for those containing double blanks) in the 96-well plate (choose carefully to optimize heating, volume, and adsorption properties), add 25µl (per 200µl sample) internal standard working solution (Concentration: 0.1 - 1ng/µl)
2) To each well/vial, add an aliquot (200-1200µL) of the applicable sample (i.e., patient specimen, standard, QC or blank)
3) To each well/vial, add 25µl (per 200µl sample) ß-Glucuronidase in 0.1M pH 4.5 NH_4OAc buffer
4) Seal plates/vials (heat-sealed foil preferred on plates)
5) Mix gently and heat at 60°C for approximately 3 hours
6) Alternatively, consider uaing prified recombinent ß-Glucuronidase at 55°C for 30 minutes.
7) Centrifuge for approximately 5 minutes at approximately 2000g

ITSP – SPE Solvent Preparation

Wash Solvent: MeOH (Honeywell part no.: 230-4 (VWR)) with 0.2% NH_4OAc.

Conditioning Buffer: trace analytical grade water fresh from a Millipore Integral Water Purification System w/BioPak (or equivalent) buffered with 20% solid NH_4OAc (atomic analysis grade, Aldrich part no.: 372331-100G). Do not use buffer from ß-Glucuronidase step 3 above.

Buffered Elution Solvent: MeOH with either 2% HOAc (atomic analysis grade, Fluka no.: 07692) when using a biphenyl LC column *or* 0.2% NH_4OAc (atomic analysis grade, Aldrich part no: 372331-100G) when using a C_{18} LC column (See the LC injection notes in the LC method below).

The ITSP Solution for RP-SPE Sample Preparation

Each sample is prepared using SPE individually for LC/MS/MS analysis. This process is performed for each well or vial (steps 7-15 continued in the next column) before moving to the next well or vial. The method operates concurrently with all successive LC/MS/MS analyses within a list, in parallel, as shown in Figure 2.

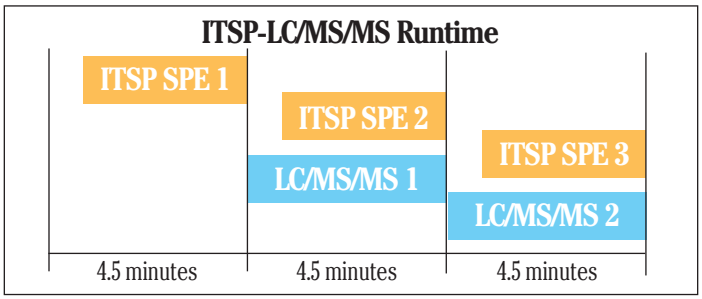


Figure 2: An ITSP solution uses the analytcal instrument's look-ahead feature embedded into the LC/MS/MS operating software to prepare samples while the instrument is analyzing the prior sample. Notice how ITSP elutes the sample just-in-time for injection.

Clean samples are eluted into 96-well plates or 2ml vials and then injected by the PAL System into the LC/MS/MS.

Reverse Phase ITSP SPE Cartridges

ITSP Product Number: 10S-UC18EC-T - United Chemical Technologies (UCT), end-capped C_{18}, 10mg, 96/Tray

Continued Step-by-Step Process

7) Buffer the urine or oral fluid using the Conditioning Buffer by adding a volume equal to 10% of the urine sample volume and mix (typically 25µL added to 250µL urine or 100µl added to 1000µl oral fluid from the Quantisal sampling device, 2% final concentration). Sufficient NH_4OAc concentration is required to achieve high recoveries for opiates. This can be performed by the PAL System or manually added to plate just prior to SPE cleanup (reseal plate if done manually).
8) Wash ITSP cartridge with 100µl Wash Solvent
9) Condition ITSP cartridge with 100µl Conditioning Buffer [steps 8-9 can be combined using the DLW option]
10) Load 200µL of urine at 5µl/s on to ITSP cartridge (match volume to sensitivity needs: 250µl perfect for most Triple Quadrupoles (QQQs), 100µl can be used with top end QQQs, up to 400µl can be used with older QQQs, see Figure 3 on following page) Alternatively, load 1000µL of oral fluid at 5µl/s on to ITSP cartridge
11) Wash the cartridge with 100µl Conditioning Buffer
12) Elute at 5µl/s with 75µl Buffered Elution Solvent into well/vial
13) Mix elution thoroughly with syringe (5x at 50% volume [minimum] at 20µl/s)
14) Inject onto LC/MS/MS system for analysis (>3x overfill recommended for precision; see LC method notes below)
15) Rinse syringe and LC injection valve with ACN/formic acid (99/1) and water/formic (99/1), cleaning should consist of at least 3 full syringe volume rinses and >1ml each pushed through the LC injection valve, then prepare (SPE) next sample in parallel to the ongoing LC/MS/MS analysis.

Recommended Work Flow:
Presume low concentrations. For the initial sample examination, load a large enough sample volume to easily hit all stated cut-offs for all drugs. Review data. In the few cases where high dose drugs are detected (exceeding the upper end [+20%] of the valid LC/MS/MS method range), the same samples can be rerun using small volume loading (25µl diluted to 250µl with Conditioning Buffer and 100µl loaded on cartridge using the PAL System) to bring the response into the valid measurement range (where needed). This approach is desirable in the clinical lab to avoid the introduction of error through manual dilution. Also, where desirable, the PAL System can be used for adding internal standard to these lower-volume samples.

PAL System Configuration: DLW2 Option with 500µl FEP sample loop and 22g needle for urine only or 1000µl FEP sample loop and 22g needle for oral fluid and urine samples.

Sample loading in ITSP SPE: The ideal way to adjust assay sensitivity via sample amount

The following figure shows the LC/MS/MS drug response as a function of urine sample volume. The linear response range for 71 pain management drugs (drugs and ISs spiked 500ng/g) is 100 to 500µl delivered with a 500µl syringe.

Note that cartridge capacity has not been completely depleted at 500µl of urine (benzos, opioids, and THCA continue to be adsorbed beyond 500µl). This, in part, can be mitigated by using lower concentration internal standards for benzos/opioids and/or increasing the amount (concentration) of NH_2,OAC added to sample. This also may be an important consideration if it is desirable to increase the number of drugs to ≥100. If one aims to measure ≥100 drugs, then use of the 30mg ITSP cartridge (Product number: 30S-UC18EC-T) with 100µl elution Buffered Elution Solvent is recommended. This same approach (30mg ITSP cartridge) also can be used for trace drug analysis (<1ng/g) when loading 400-1200µl urine.

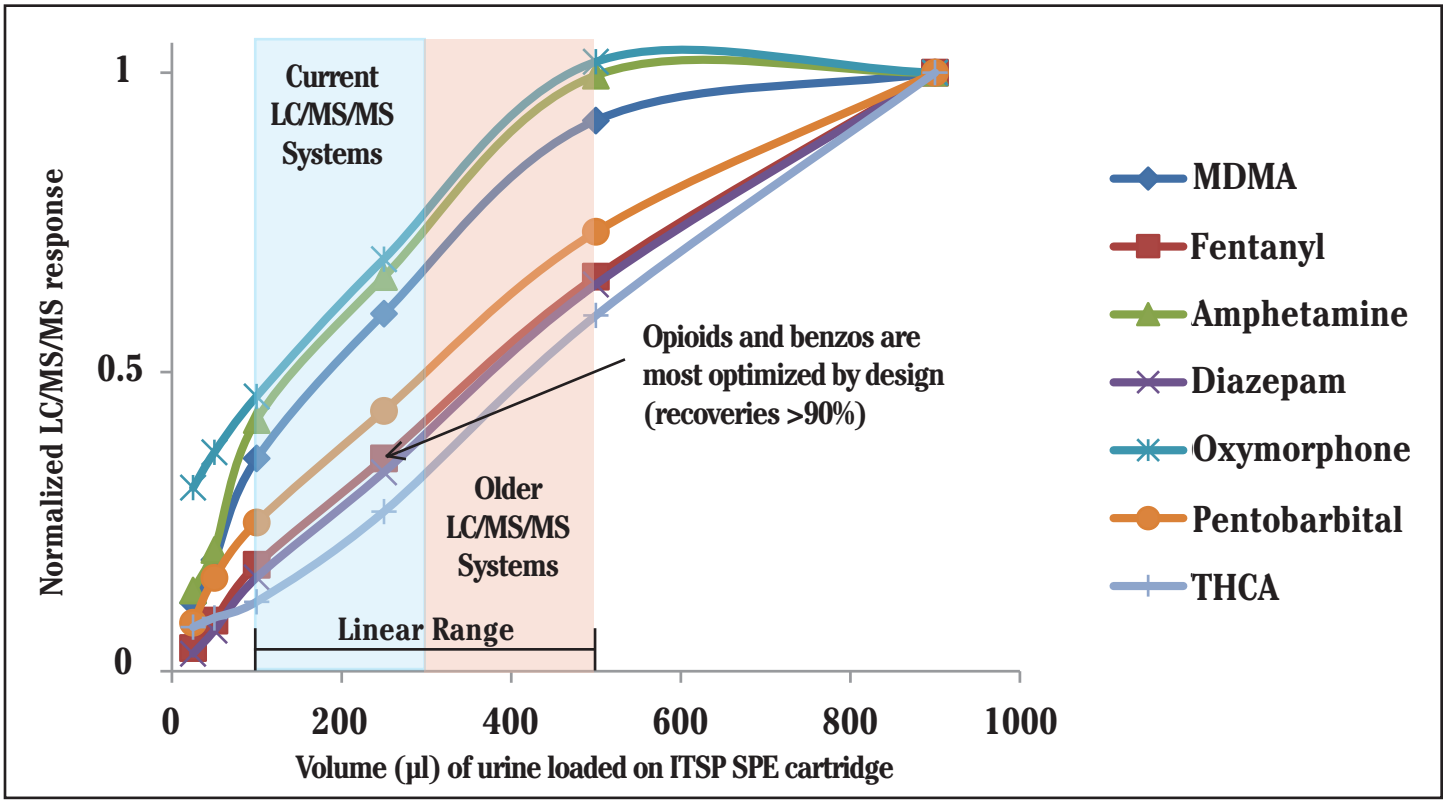


Figure 3: This illustration shows how the same analytical method can be used aross a variety of instrument sensitivity ranges. By adjusting the sample load onto ITSP SPE cartridges, less sensitive instruments can achieve excellent analysis of even the most difficult samples.

LC/MS/MS conditions to cover a wide range of drugs after ITSP RP/SPE

Mobile Phases:

A – Trace analytical grade water fresh from a Millipore Integral Water Purification System w/BioPak (or equivalent) buffered with 1% HOAc (atomic analysis grade, Fluka product no.: 07692)

B – ACN (LC/MS grade, Honeywell product no.: 015-4)

Column:

GL Sciences Inertsil C_{18} ODS3, 3µm particles, 2.1 x 50mm or same size biphenyl column (Restek Raptor) held at 50°C using a heat exchanger in column oven to preheat mobile phase.

Gradient (1 ml/min):

Time = 0.00: 97% A, 3% B (start)
Time = 0.05: 97% A, 3% B (hold)
Time = 0.60: 80% A, 20% B (linear gradient)
Time = 3.00: 40% A, 60% B (linear gradient)
Time = 3.20: 5% A, 95% B (linear gradient)
Time = 3.35: 5% A, 95% B (hold)
Time = 3.36: 100% B (column clean up)
Time = 3.50: 100% B (column clean up)
Time = 3.51: 97% A, 3% B (column conditioning)
Time = 4.00: 97% A, 3% B (column conditioning)

Depending on the drugs measured, gradient transition points and/or ramp rates may have to be adjusted to separate isobaric drugs and/or to achieve at least 16 data points across each LC peak. These (and all LC/MS/MS parameters) should be optimized, fully functional, and made routine based on solvent/standards-only solutions prior to proceeding with on-line SPE with ITSP.

Sample Injection by PAL System Relative to Mass Spec Sensititvity

1) Fill valve/loop with trace analytical grade water prior to injection.
2) In all cases, a pre-cut and polished SS loop is used.
3) Using ordinary LC/MS/MSs: 5µl loop using >3x overfill. If peak shape and/or retention are insufficient for early eluting peaks, check buffering in SPE eluent (see Figure 4 below).
4) Using top-end LC/MS/MSs: 1-2µl loop using >3x overfill (use narrower LC peaks to improve speed and/or separation).
5) Injection volume should be held to a maximum of 5µl when using a 2.1mm diameter LC column. Larger volumes increase peak area primarily in width, not height, and thus deteriorate the LC separation with little, if any, gain in sensitivity. Always operate LC/MS/MS at optimal conditions.
6) Chemical presentation of the sample from ITSP SPE to the LC is important. In LC analysis, control of the pH (ionization state) controls peak shape (see Figure 4). Elution in 80% ACN limits LC injection volume to ~2µL (2.1 x 50mm column). Elution in 100% MeOH (buffered) allows 5µL LC injection. Viscosity has an equally important role in LC injection along with pH.

MS/MS Conditions:

Use all the usual MRMs for all drugs. Barbiturates and THCA use (–) ion MRMs (Figure 5), all others (+) ion.

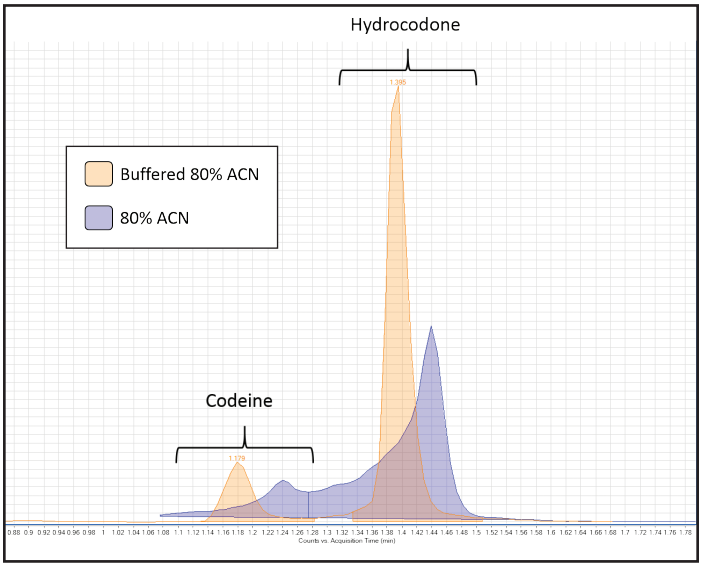


Figure 4: This chromatogram illustrates two ITSP SPE elutions, one using a buffer and the second without.

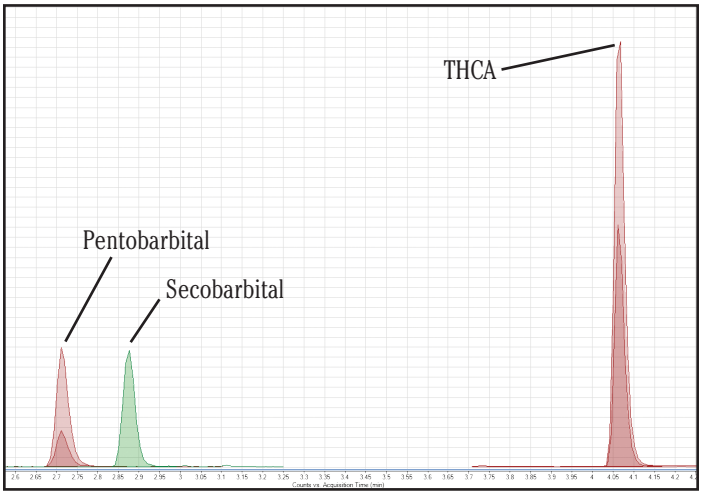


Figure 5: This chromatogram illustrates that YES, acidic drugs can be measured by LC conditions used for basic drugs.

The mechanics of ITSP's patented cartridges for solid phase extraction

ITSP Solutions' patented cartridge for automated SPE uses precise control of flow rates and volumes for solvents and samples through the sorbent by the PAL System while the LC/MS/MS or GC/MS/MS is analyzing a sample in parallel.

The PAL System uses ordinary autosampler tray holders, trays, syringes and solvent reservoirs to which we adapt our single position solution to automate SPE, the gold-standard for sample preparation for Liquid and Gas Chromatography.

By aspirating a solvent or sample, then penetrating the septum on the ITSP cartidge, the syringe plunger can pass both through the sorbent bed at higher precision than achievable by ordinary manual and automated vacuum-based and pneumatic systems (none of which have flow control). Figure 6 details the construction of the ITSP cartridge.

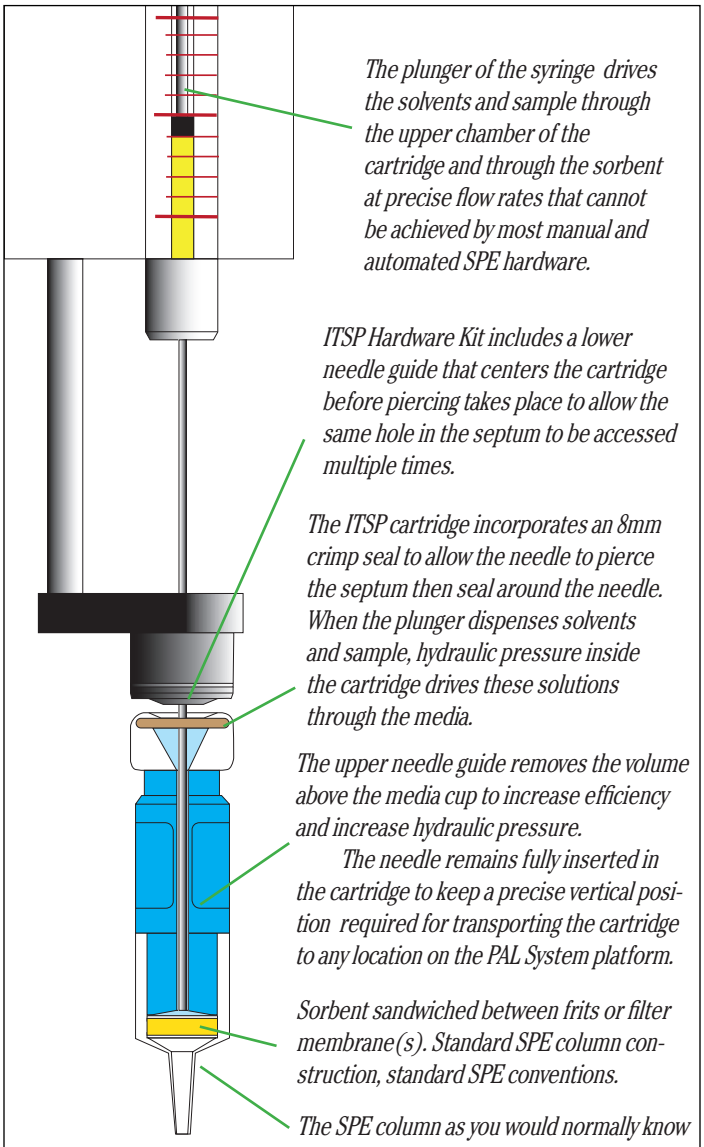


Figure 6

ITSP uses PAL Systems hardware, the most commonly used autosampler for GC/MS/MS and LC/MS/MS worldwide, and is fully able to seamlessly integrate into all LC/MS/MS and GC/MS/MS software systems.

Whereas some ITSP templates (provided at no cost with the PAL-xt hardware kit) are standard scripts supplied with new PAL RTC Systems, a high degree of customization is possible, including bar code reading (chain of custody), all sample formats (96 & 384 well plates / vials / 12 x 75mm tubes), filtration, and multidimensional SPE.

The PAL RTC

The CTC Analytics PAL RTC (Figure 7) can change between up to six different syringe options (1µl to 10ml) automatically. The Dilutor Tool speeds up the conditioning/elution steps.

This RTC is shown with a single location for 96 ITSP SPE samples. There is ample space on the rail to add additional ITSP stations to expand the number of samples per sample run.

ITSP Solutions, Inc. SPE sample preparation was used to introduce this platform at ASMS in 2013. All ITSP objects and template methods are embedded and/or provided by CTC Analytics Value-Added Resellers (VARs) and by many major analytical instrument manufacturers (OEMs) globally.

The PAL-xt with DLW2 Option

The CTC Analytics PAL-xt Systems (Figure 8) are the most-used autosamplers in the market. The flexibility, precision and dependability of the instrument are unmatched and are therefore sold by all OEMs and CTC's global network of VARs.

When coupled with an ITSP solution laboratories can increase the efficiency of the lab by automating labor-intensive sample preparation processes onto an instrument that must already be purchased for automation of an LC or GC analytical instrument.

ITSP recommends adding (if it isn't already equipped with one) the Dynamic Load and Wash (DLW) option. This allows for more efficient solvent dispensing, and most importantly, cleaning of the syringe and valve inlets to almost completely eliminate carryover. ITSP operates during the LC/MS/MS analysis of a sample to extract compounds of interest from the next sample. There is no carryover associated with ITSP solutions for sample prep and no loss of the chain of custody. The PAL System injects the eluate onto the instrument and cleans the syringe and the valve inlet before preparing the next sample. Chain of custody is enhanced due to the removal of manual interaction.

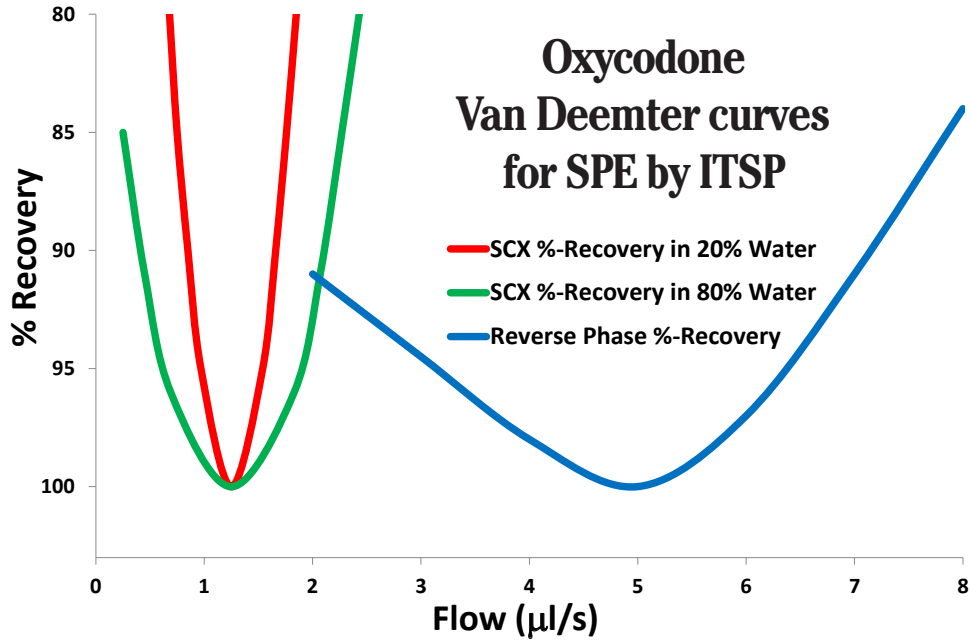
PAL SYSTEM
Ingenious sample handling
www.PALSystem.com

Figure 7: The PAL RTC pictured here is outfitted with product number: HW3-KIT which includes a single 96-position ITSP Solid Phase Extraction workstation (ITSP lower needle guide for a single RTC Syringe tool of your choice, a 3-position tray holder and a waste receptacle with hose bib). Unlike other dedicated on-line SPE systems, ITSP uses standard CTC trays and occupies only one position on the deck, allowing for other applications to run using the PAL RTC as well.

Figure 8: The PAL-xt with DLW2 option pictured here is outfitted with HW-KIT-APM which includes two 96-position ITSP Solid Phase Extraction workstations (ITSP lower needle guide, three 2-position tray holders, two 3-position solvent reservoirs and two waste receptacles with hose bibs). As with the RTC above and unlike other dedicated on-line SPE systems, ITSP uses standard CTC trays and occupies only two positions on the deck, allowing for other applications to run using the PAL System as well.

SPE is Chromatography!

Optimized outcomes require accurate flow



SCX optimum velocity = 0.37 mm/s (1.2 µl/s by ITSP) with little room of error!
Reverse Phase optimum velocity = 1.5 mm/s (5 µl/s by ITSP) 4x higher than SCX!
SPE flow driven pneumatically or by vacuum cannot achieve and maintain optimum flow!

Assurance Scientific Laboratories

727 Memorial Drive, Suite 103, Bessemer, Alabama 35022 • 855-319-4459

www.AssuranceScientific.com

ITSP® SOLUTIONS, INC.

SmartSPE™

212 Northlake Drive, Hartwell GA 30643 • 10 South Carolina Street, Hartwell, GA 30643
855-395-8300 Toll-Free • 706-395-8300 International • 706-395-4946 FAX

www.ITSPsolutions.com

US Patents: 6,859,615 & 7,001,774 • European Patents: EP 1 174 701 • Canadian Patent: 2,316,648