## HPLC 2010
# Newsletter
BOSTON

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June 2010

## More information about DPX

## Automated Vortex / Centrifuge for MPS

*GERSTEL is presenting the novel Vortex and Centrifuge option for the GERSTEL MultiPurpose Sampler (MPS) at HPLC 2010.*

Sample preparation steps such as liquid-liquid extraction can now be performed faster and more efficiently with the benefit of improved phase separation by using vortexing and medium level centrifugation, for example when performing QuEChERS clean-up. Replaceable rotors for 2 mL or 10 mL vials are available. For more information, please visit the GERSTEL booth.
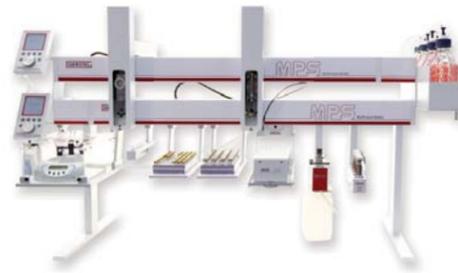
## GERSTEL LC/MS Effluent Optimizer (LEO)

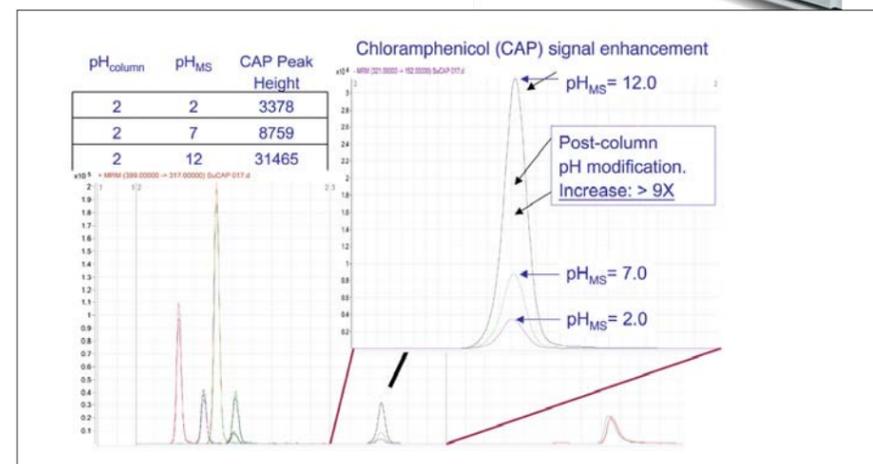In LC/MS, we work hard to reach the goal of achieving the perfect LC separation and combining it with the most efficient ionization and lowest achievable MS detection limits for our analytes.

The LC separation may require a certain pH and polarity range of the eluent, while analyte ionization in the LC/MS ionization source requires yet another pH, a different buffer – or even derivatization of the analyte for best possible efficiency or optimized spectral information. How to optimize both? The logical answer is to take the effluent from the perfect LC separation and optimize it for MS analysis. This task is easily possible when you add the GERSTEL LC/MS Effluent Optimizer (LEO) module to your LC/MS/MS system. Application examples show sensitivity gains of up to a factor of 40 by simply adding a salt solution to the LC effluent and/or changing its pH. The LEO module is quickly and easily installed in your LC/MS system. A solvent mixture, buffer solution or reagent is then easily added to the effluent ensuring that the LC separation can be performed under optimal conditions while also enabling maximum yield in the MS ionization process, helping to reduce or eliminate Ion Suppression. Whether you are looking to perform pH adjustment or post-column derivatization, for method development or routine analysis, when you use LEO and the GERSTEL MAESTRO software you can easily and efficiently control all parameters as part of the overall method. Just one sequence table controls the entire system from sample preparation through LC separation and effluent optimization to MS analysis. It is all done with the click of a mouse.

| pH_column | pH_MS | CAP Peak Height |
|---|---|---|
| 2 | 2 | 3378 |
| 2 | 7 | 8759 |
| 2 | 12 | 31465 |

Chloramphenicol (CAP) signal enhancement

$pH_{MS} = 12.0$

Post-column pH modification. Increase: > 9X

$pH_{MS} = 7.0$

$pH_{MS} = 2.0$

Enhancing signal in LC/MS through post-column pH modification. Example: Chloramphenicol (CAP)

**Automated Sample Preparation**

# Doing Drugs? II

Comprehensive Analysis of Drugs of Abuse in Urine with Automated Disposable Pipette Extraction (DPX) and HPLC/MS/MS with dynamic MRM.

In order to analyze biological specimens for drugs such as benzodiazepines, it is necessary to perform sample preparation to eliminate matrix interference and ion suppression. Solid-phase extraction is generally the preferred sample preparation technique, in this study Disposable Pipette Extraction (DPX) was utilized. DPX is a fast dispersive solid-phase extraction technique that uses loosely contained sorbent in a disposable pipette tip. The sample is aspirated into the tip where it is actively mixed with the sorbent and forms a suspension. The main advantages of the DPX technology are that the extraction is rapid, minimal solvent waste is generated, and the entire process can be fully automated including introduction of the extract to the chromatographic system. The GERSTEL MPS autosampler performs DPX extractions in approximately 5 minutes using reversed phase (DPX-RP), cation exchange (DPX-CX) or immunoaffinity sorbent material. For determination of target drugs, GC/MS or HPLC/MS/MS are generally the preferred techniques. The advantage of LC/MS/MS is that chemical derivatiza ➔

## Automated DPX process

All steps are performed automatically by the MPS.
If needed, the sorbent is conditioned with solvent prior to the extraction process.

**1** Sample is drawn into the pipette tip for direct contact with the solid phase sorbent. There is no contact between the sample and the syringe used to aspirate the sample and therefore no risk of cross contamination.

**2** Air is drawn into the pipette tip from below through the frit. Turbulent air bubble mixing creates a suspension of sorbent in the sample, ensuring optimal contact, highly efficient extraction, and high recovery.

**3** The extracted sample is discharged, typically after 30 seconds.
If needed, the sorbent can be washed to remove unwanted residue.
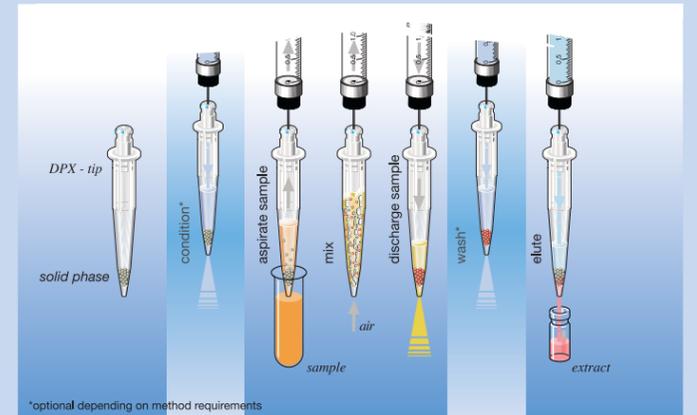
**4** Extracted analytes are eluted using a suitable solvent, which is added from above for most efficient elution. The eluate is collected in a vial for subsequent sample introduction to LC/MS or GC/MS.

The total time required for extraction in the examples shown in this article was always less than 6.5 minutes. Sample preparation and GC/MS or LC/MS determination can be performed in parallel for best possible throughput and system utilization.

DPX - tip
solid phase
condition*
aspirate sample
mix
discharge sample
wash*
elute
sample
air
extract
*optional depending on method requirements
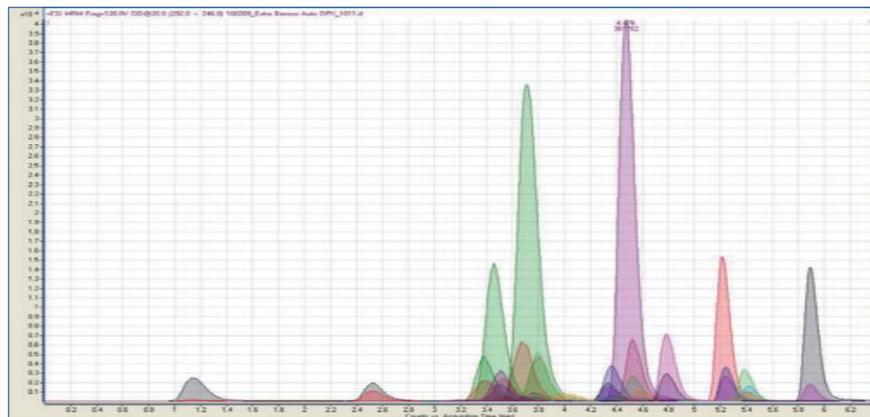
Extracted Ion Chromatogram of DPX extracts of urine samples spiked at 7.5 ng/mL.



triazolam - 8 Levels, 8 Levels Used, 16 Points, 16 Points Used, 17 QCs
y = 0.0085 * x − 7.9111E-004
R^2 = 0.97829668

clonazepam - 8 Levels, 8 Levels Used, 16 Points, 16 Points Used, 17 QCs
y = 0.0227 * x − 0.0014
R^2 = 0.99739123
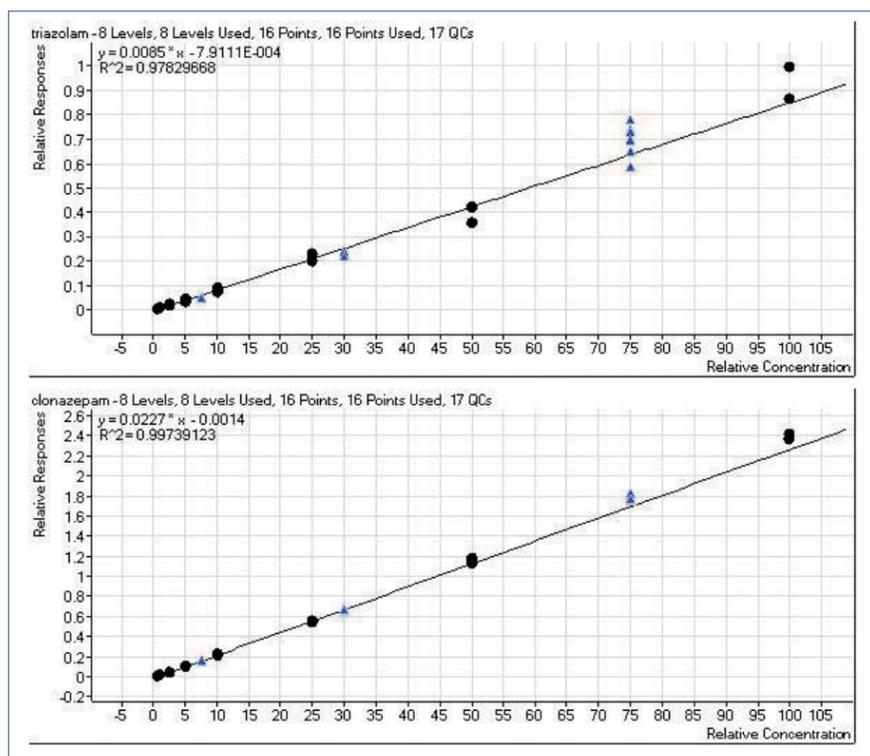
Representative Calibration Curve of Triazolam and Clonazepam.

| Compound | R² | LOQ [ng/mL] |
|---|---|---|
| temazepam[C] | 0.9917 | 0,5 |
| alprazolam[C] | 0.9901 | 0,5 |
| oxazepam[C] | 0.9950 | 0,5 |
| lorazepam[C] | 0.9908 | 0,5 |
| estazolam[D] | 0.9955 | 0,5 |
| α-OH-alprazolam[A] | 0.9958 | 0,5 |
| flurazepam[B] | 0.9937 | 0,5 |
| midazolam[B] | 0.9919 | 0,5 |
| flunitrazepam[E] | 0.9959 | 0,5 |
| diazepam[B] | 0.9969 | 0,5 |
| clonazepam[E] | 0.9974 | 0,5 |
| clobazam[E] | 0.9947 | 0,5 |
| bromazepam[E] | 0.9973 | 0,5 |
| triazolam[C] | 0.9783 | 0,5 |
| nordiazepam[B] | 0.9941 | 0,5 |
| nitrazepam[C] | 0.9910 | 0,5 |

Calibration Curve Results Calculated using d₅-α-Hydroxyalprazolam (A), d₅-Nordiazepam (B), d₅-Oxazepam (C), d₅-Estazolam (D) and d₄-Clonazepam (E).

Recovery for 16 benzodiazepines using our DPX-LC/MC/MS-Method. The low recovery of clobazam is based on its low extraction efficiency. Unlike the other benzodiazepines it does not contain a tertiary amine in its chemical structure and therefore binds less tightly to the DPX-CX cation exchange material. This result supports the specifity of the DPX-CX sorbent for tertiary amines.

| Compound | Recovery [%] |
|---|---|
| Alprazolam | 65 |
| α-OH alprazolam | 100 |
| Clonazepam | 91 |
| Diazepam | 80 |
| Flunitrazepam | 86 |
| Lorazepam | 49 |
| Nitrazepam | 93 |
| Nordiazepam | 91 |
| Oxazepam | 91 |
| Temazepam | 84 |
| Estazolam | 90 |
| Clobazam | 6 |
| Triazolam | 54 |
| Flurazepam | 82 |
| Midazolam | 57 |
| Bromazepam | 43 |

tion of the analytes is not required, making sample preparation simpler and less time consuming. In addition, highly efficient ionization, in combination with tandem mass spectrometry results in high sensitivity and selectivity. This study focused on performing automated extraction of reduced sample volumes coupled with LC/MS/MS to provide high throughput analysis "one sample at a time". The sample preparation time was decreased sufficiently to allow the extraction of a sample during the chromatographic analysis of the previous sample in the sequence.

## Experimental

**Instrumentation**: All Analyses were performed using an Agilent 1200 HPLC with an Agilent 6410 Triple Quadrupole Mass Spectrometer with ESI source. Sample Preparation and Sample Introduction was performed using a GERSTEL MultiPurpose Sampler (MPS XL).

## Materials

Benzodiazepine Multi-Component Mixture 8, containing Clonazepam, Temazepam, Nitrazepam, Alprazolam, Diazepam, Flunitrazepam, Lorazepam, and Oxazepam at 250 μg/mL each in acetonitrile, Nordiazepam, Clobazam, Bromazepam, Estazolam, Flurazepam, Midazolam, and Triazolam, at 1.0 mg/mL each in methanol, and α-hydroxyalprazolam, at 100 μg/mL in methanol, were purchased from Cerilliant. Intermediate stock solutions of the sixteen benzodiazepines were prepared in water from appropriate dilutions of these stocks.

Deuterated analogues d5-nordiazepam, d5-α-hydroxyalprazolam, d5-oxazepam, d4-clonazepam, and d5-estazolam, at 100 μg/mL each in methanol, were purchased from Cerilliant. These stocks were combined and diluted with water to be used as internal standards during analysis.

### Urine Sample Pretreatment

- Pipette 260μL of hydrolyzed urine sample into a clean 12 x 75mm culture tube.
- Pipette 250μL of 1.0M HCl into the tube and vortex mix for a few seconds.
- Pipette 250μL of acetonitrile into the tube and vortex mix for a few seconds.
- Filter the sample using an Agilent 2 in 1 syringe filter and collect the filtrate into a clean 12 x 75mm culture tube.
- Place the filtered urine sample onto the GERSTEL MPS 2XL multi-purpose sampler with DPX Option.

### Whole Blood Sample Pretreament

- Pipette 200μL of whole blood sample into a clean 12 x 75mm culture tube.
- Pipette 800μL of acetonitrile into the tube and vortex mix for a few seconds.
- After centrifugation for 10 minutes to pellet the precipitated proteins, transfer the supernatant into a clean 12 x 75mm culture tube.
- Place the sample onto the GERSTEL MPS 2XL multi-purpose sampler with DPX Option.

## Extraction

A GERSTEL MultiPurpose Sampler (MPS) was set up with 1 mL DPX-CX tips (DPX Labs, LLC, Columbia, SC) for extraction of hydrolyzed urine samples. The following automation method was used: 250 μL of 30% acetonitrile/water was slowly added through the top of the DPX tip at a rate of 50 μL/s to wet the sorbent. The

### Analysis Conditions HPLC

| Column: | Zorbax Eclipse, XDB C18 RRHT (2.1 mm x 50 mm, 1.8 μm) |
|---|---|
| Mobile Phase: | A – 20 mM ammonium-formate, pH 8.6 B – 10 % isopropyl alohol in methanol |

Gradient:

| Time (min) | Flow (mL/min) | Pressure (bar) | % B |
|---|---|---|---|
| 0 | 0.2 | 600 | 45 |
| 2 | 0.2 | 600 | 45 |
| 5 | 0.2 | 600 | 95 |
| 9 | 0.2 | 600 | 95 |
| 9.5 | 0.2 | 600 | 45 |

| Column temperature: | 35 °C |
|---|---|
| Injection volume: | 2,5 μL |
| Run time: | 10 min |

**MS Parameters**

| Ionisation: | ESI positive ion mode |
|---|---|
| Gas Temperature: | 350 °C |
| Gas Flow (N2): | 12 L/min |
| Nebulizer pressure: | 35 psi |
| Capillary: | 4,5 kV |

sample was then aspirated into the DPX tip and mixed with the sorbent by drawing in an additional 1.3 mL of air. After a 20 s equilibration time to allow analyte binding, the resulting solution was dispensed to waste. To wash off excess matrix, 500 μL of a 10% acetonitrile/water wash solution was added to the sorbent material through the top of the DPX tip and dispensed to waste followed by an additional wash using 500 μL of 100% acetonitrile. For elution of the analytes, 700 μL of 78/20/2 (v/v) of methylene chloride/isopropanol/ammonium hydroxide was added to the sorbent material through the top of the DPX tip and the eluate dispensed directly into a clean 2 mL autosampler vials. All eluents were dried and reconstituted with 50 μL of water before injection.

## Results and Discussion

The DPX-CX extractions were readily performed using the GERSTEL MultiPur-
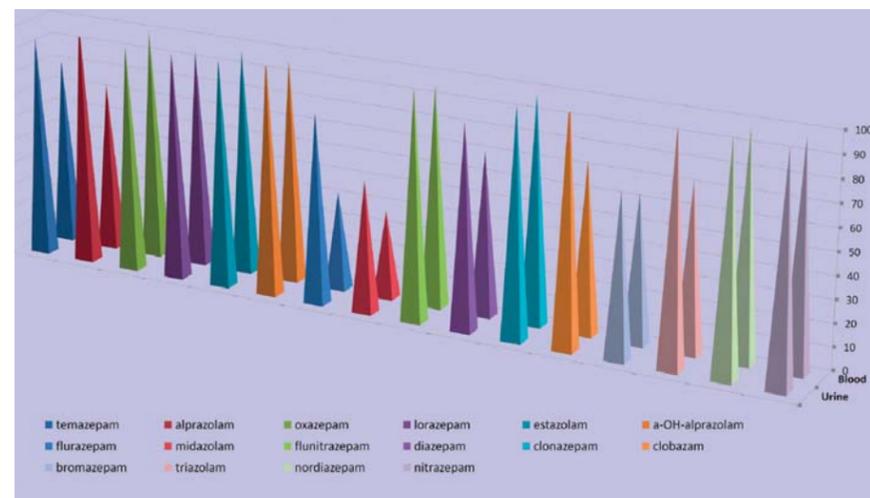


MPS 2XL MultiPurpose Sampler with the GERSTEL DPX Option.

pose Sampler. The entire extraction process took approximately 6.5 minutes per sample. Because a basic eluent is used with the cation exchange sorbent, the eluents had to be solvent exchanged into the HPLC mobile phase. In light of the world-wide acetonitrile shortage, it was found that the use of 10% isopropyl alcohol in methanol as the organic modifier of the mobile phase provided an alternative to using acetonitrile. All HPLC/MS spectra were collected using dynamic multiple reaction monitoring (MRM) providing good sensitivity for the analysis of these drugs at low concentrations. A rapid resolution HPLC column was chosen in order to perform the chromatographic data in approximately 10 minutes.

## Conclusion

Automated DPX extraction of benzodiazepines from urine can be performed successfully using the GERSTEL MPS. In the work presented here, the total extraction time was 6.5 minutes, The total sample preparation time was less than the chromatographic run time, which means that the next sample can be prepared with separation of the current sample is in progress. Whenever the LC/MS/MS system has finished a run, the next sample is ready to be introduced ensuring the highest possible throughput. Additionally, "just in time" sample preparation helps to ensure that the prepared sample is not kept in the autosampler for a long time prior to being analyzed, reducing the risk of analyte degradation and helping to maintain sample integrity.

Using this DPX-LC/MS/MS method for determination of benzodiazepines in urine high recoveries for all benzodiazepines were achieved. The calibration curves showed good linearity (R² ≥ 0.98) with limits of quantitation of 0.5 ng/mL. The method proved to be accurate and precise. Accuracy data averaged 102% (range: 84.9% - 136%) and precision data averaged 5.48%RSD (range: 1.01% –26.4%) for all benzodiazepines analyzed.

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Matrix effects on the ionization of benzodiazepines in hydrolyzed urine and whole blood compared to the neat standard solutions in pure water (100%).