

# **DIRECT INJECTION OF DRUG SCREENING RACE HORSE SERUM AND URINE SAMPLES BY HT MDLC MS**

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# INTRODUCTION

Due to the high stakes involved in horse racing, blood and urine samples are taken from the top 3 finishers at every state sanctioned race in California. These samples must be analyzed for a wide range of drugs before the final winnings are disbursed.

Current methods require off line cleanup and screening by LC-MS or GC-MS and positive hits must be confirmed and quantitated (MS/MS).

This study will look at a Multi-Dimensional LC system that allows direct injection of serum and urine samples on to a denaturing size exclusion chromatography (DSEC) column to remove all of the high (>2000) MW matrix components which normally interfere with MS analyses. The MW range from 200-2000 containing the drugs of interest is heart cut from the D1 DSEC, desalted and analyzed by ballistic gradient LC with an API-TOF MSD for screening and a API-TOF MS/MS for confirmation and quantitation.

# EXPERIMENTAL

MDLC		Michrom Paradigm MS4B 100% Metal Ion Free Fluid Path
Columns	DSEC	4.6 x 100 mm Michrom DSEC
	Ballistic RP	Micro Magic C18 Bullet
MS	Screening	Michrom Paradigm MX1 API TOF MSD
	Confirm	Thermo TSQ Quantum API Triple Quadrapole MS/MS

# HT MDLC API-TOF MSD SYSTEM



# ONLINE PREP OF PHYSIOLOGICAL FLUIDS

Although MS/MS allows very selective analysis of drugs, sample matrix interferences can clog the chromatography column and/or contaminate the MS source (which impacts throughput and robustness), as well as causing signal suppression (which impacts quantitation). In physiological fluids, the major interferences are molecules over 2000 MW (proteins) and less than 200 MW (salts).

In DSEC, the mobile phase solubilizes all matrix components, dissociates protein-drug interactions, eliminates secondary interactions (charge, hydrophobicity) and allows true separation by MW. By careful choice of column properties (physical and chemical) and mobile phase components (organic, chaotropes, etc) a DSEC online prep of equine samples was achieved in 2-5 minutes.

# OPTIMIZED DSEC BUFFER AND COLUMN

The Michrom DSEC buffer used in this study was developed specifically for this application after a great deal of trial and error to find conditions that eliminated all chemical interactions (column-protein, column-analytes, protein-protein and protein-analytes) and give a true separation based only on molecular size.

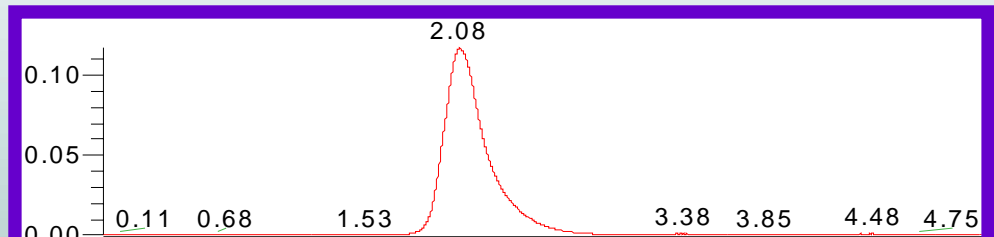
The DSEC column needs to have good chemical and physical stability, high sample capacity, proper pore size distribution for adequate resolution, no secondary interaction sites (ionic, hydrophobic, etc) and the ability to perform reliably and robustly for thousands of high throughput sample runs. The Michrom DSEC column used in this study was developed specifically for this application and has been shown to meet all of these criteria, when used with the Michrom DSEC buffer system. Examples of the separation of large proteins and small molecules are shown in the following slide.

# PROTEIN & DRUG SEPARATIONS BY DSEC

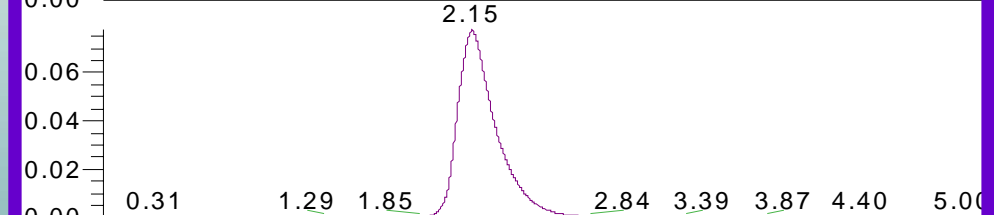
4.6x100mm DSEC Column  
DSEC Buffer System

UV<sub>280</sub>  
1 ml/min

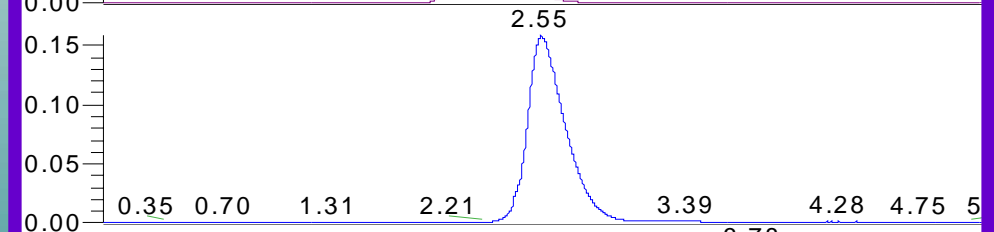
Immuno g-Globin (150 kD)



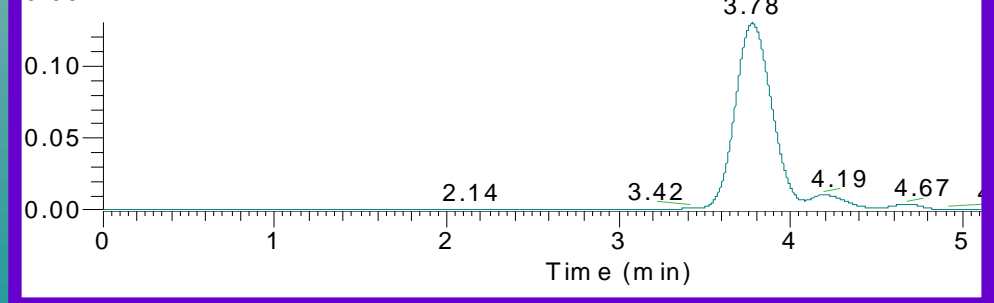
Serum Albumin (69 kD)



Insulin (6 kD)



Drugs (0.2-1.2 kD)



# REPRODUCIBILITY OF DSEC FOR SERUM

To test the robustness of this method, horse serum and urine samples were spiked with a mixture of small molecule standards (0.2 – 1.2 kD) and diluted 50:50 with a 2X concentrate of the DSEC buffer (this helps to keep all matrix components in solution and dissociates any molecular interactions). The samples were then loaded into 6 x 96 well plates for continuous analysis over 10 x 24 hour days.

The slide that follows shows the excellent reproducibility of this method over 5 days for the serum separations, and we saw no deterioration in the separation, change in retention or increase in column back pressure after injection of 1440 serum and 1440 urine samples.

# REPRODUCIBILITY OF DSEC FOR SERUM

4.6x100mm DSEC Column      UV<sub>280</sub>  
DSEC Buffer System      1 ml/min

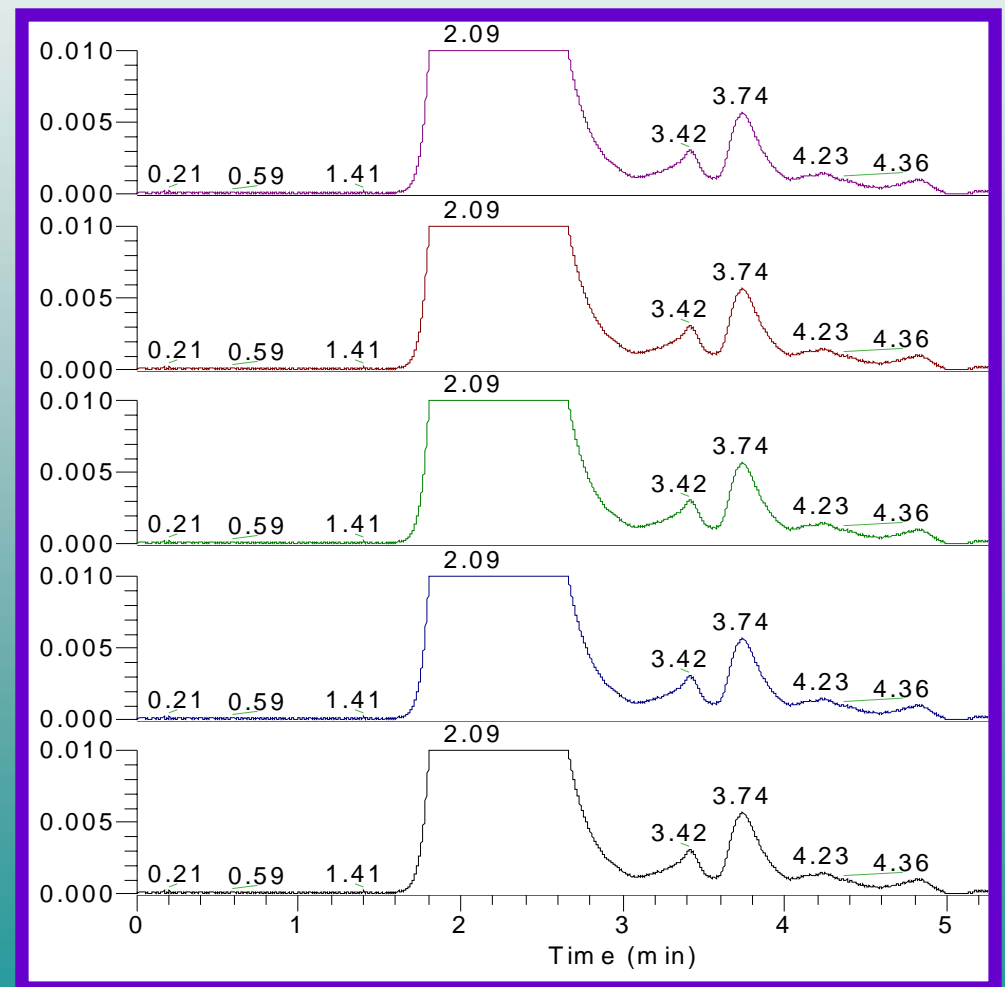
100 ul Horse Serum + 1 ng Drugs  
Day 1 - Run 144

100 ul Horse Serum + 1 ng Drugs  
Day 2 - Run 432

100 ul Horse Serum + 1 ng Drugs  
Day 1 - Run 720

100 ul Horse Serum + 1 ng Drugs  
Day 1 - Run 1008

100 ul Horse Serum + 1 ng Drugs  
Day 1 - Run 1296



# INCREASING THE SPEED OF DSEC

As the DSEC online prep is run in parallel with the D2 ballistic gradient RPLC MS analysis, these 5 minute DSEC runs allowed us to process 288 samples per day (3 x 96 well plates). Since the Paradigm AS2 HT Autosampler used in this study can hold 6 x 96 well plates, we decided to try doubling the speed of the analysis.

At a flow rate of 2 ml/min, the DSEC separation was complete in 2.5 minutes and adequate resolution was still achieved between the proteins and the small molecule analytes of interest, as shown on the slide that follows. A second week of running this assay at the higher flow rate gave similar results, with no loss in performance after 2880 runs of 100 ul serum samples.

# INCREASING THE SPEED OF DSEC

4.6x100mm DSEC Column      UV<sub>280</sub>  
DSEC Buffer System          2 ml/min

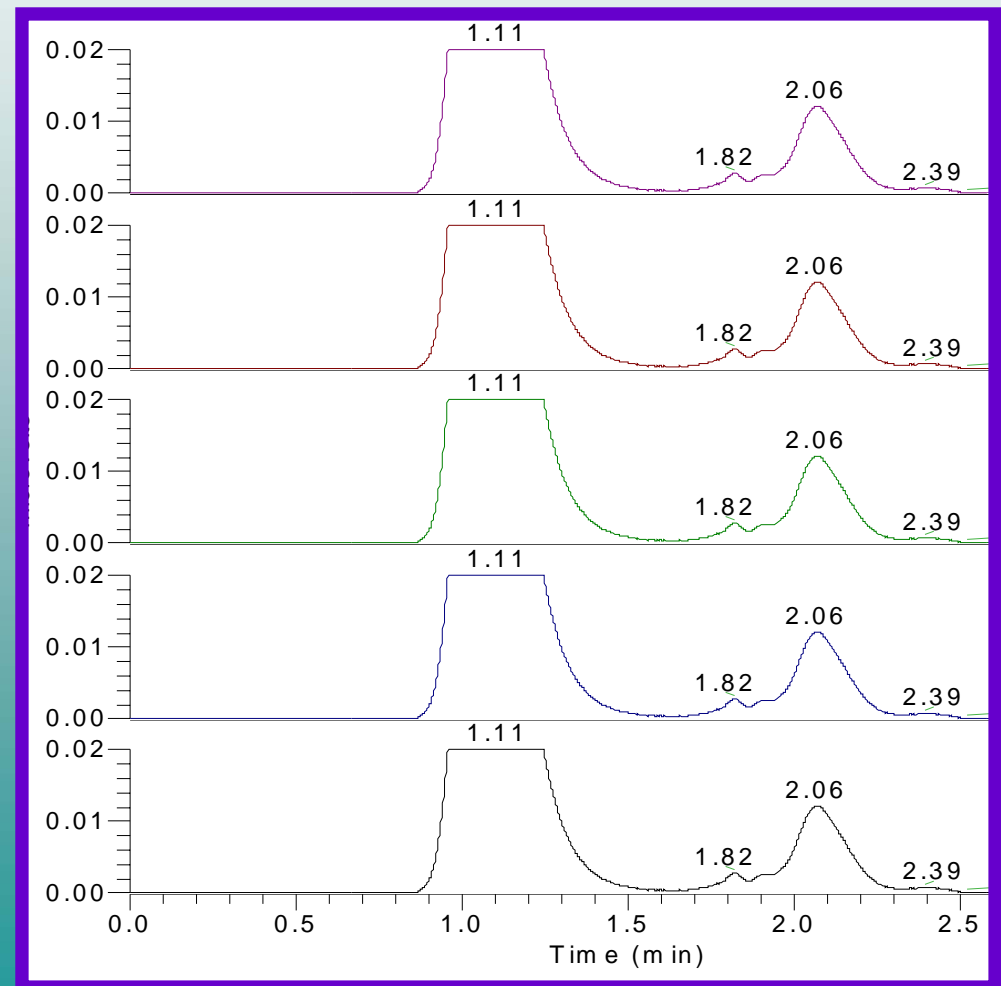
100 ul Horse Serum + 1 ng Drugs  
Day 1 - Run 244

100 ul Horse Serum + 1 ng Drugs  
Day 2 - Run 862

100 ul Horse Serum + 1 ng Drugs  
Day 3 - Run 1440

100 ul Horse Serum + 1 ng Drugs  
Day 4 - Run 2016

100 ul Horse Serum + 1 ng Drugs  
Day 5 - Run 2592



# D2 BALLISTIC RPLC MS OF ANALYTES

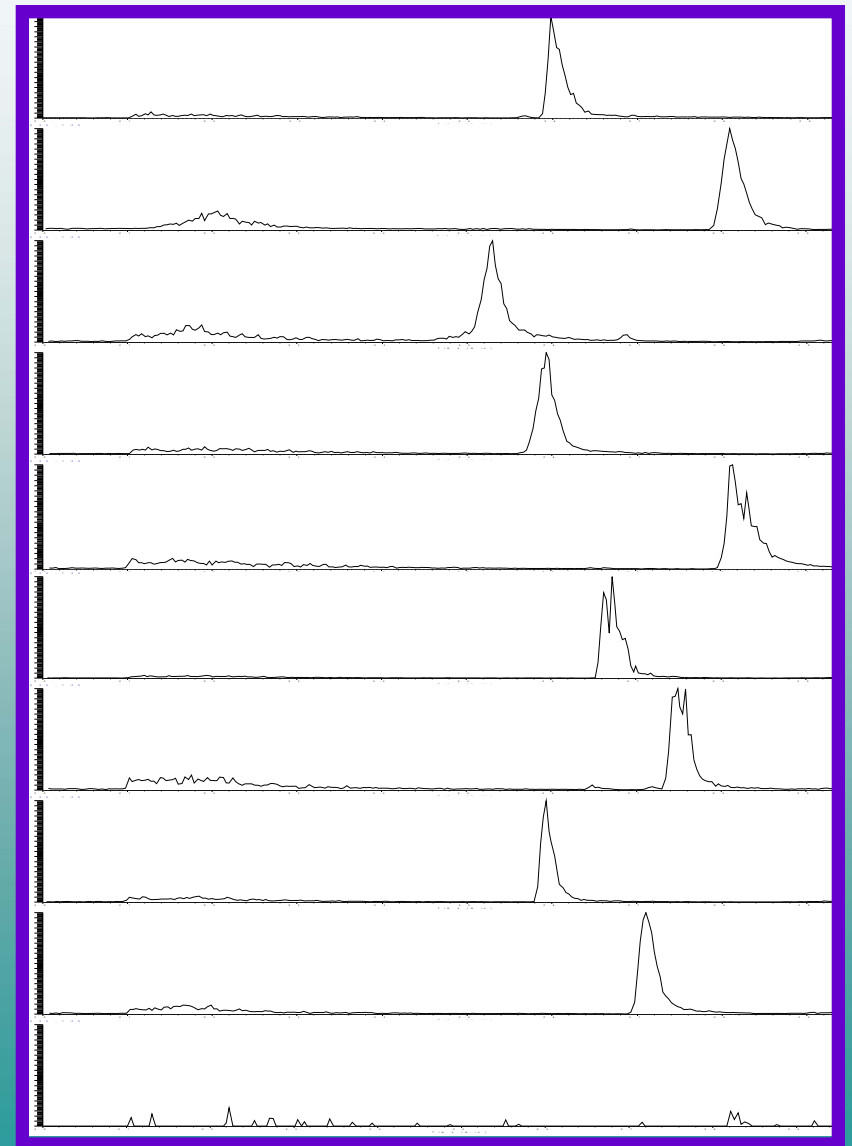
In the automated MDLC system used in this study, the D1 DSEC column eluent (D) is sent to waste for the first 1.8 min, then switched inline for 0.5 min with aqueous eluent (C), which loads, concentrates and desalts the analytes of interest on a macrotrap 1 column. At 2.5 min, the macrotrap 1 is switched inline to a D2 ballistic gradient RPLC (A/B) run on a C18 Micro Magic Bullet column into the MS in a total run time of 2.5 min.

When the macrotrap 1 column is switched inline to RPLC, macrotrap 2 is switched inline with the D1 DSEC, ready to collect the analytes of interest from the second sample, which is being separated on D1 while the first sample is being analyzed on D2. This allows parallel sample processing for a throughput of 6x96 (576) samples/24 hours.

# MDLC MS OF DRUGS IN HORSE SAMPLES

The XIC traces at the right show a horse serum sample that was spiked at 1 ng/ml with 8 known drugs of abuse (top 8 traces) and an internal standard (trace 9), as well as a blank serum sample (trace 10).

These samples were run on the DSEC-RP MDLC system in 2.5 minutes and detected using the MX1 ESI-TOF MSD. The internal standard was used for both quantitation and mass calibrations.



# SUMMARY

Due to the stakes involved in modern horse racing, it is critical to get the analysis of potential drug abuse done as quickly as possible, while still maintaining data quality that may need to stand up in court. The UCD equine testing lab currently runs a variety of sample preparation techniques prior to LC or GC MS or MS/MS, and would like to speed up and automate as much of these processes as possible.

This study introduces a powerful MDLC protocol that allows direct injection of physiological fluids into the MDLC for direct MS or MS/MS analysis. We plan to test this protocol on the hundreds of drugs we currently are required to analyze and hope that it will become the method of choice for most of our assays in the future.

# CONCLUSIONS

- DSEC/RP MDLC Allows Direct Injection of Serum and Urine
- DSEC Provides True Separation by Molecular Size
- The DSEC Buffer Eliminates Unwanted Interactions
- Drugs Can be Isolated and Analyzed in 2-5 min
- DSEC/RP MDLC is Very Robust and Reproducible
- DSEC/RP Couples With TOF-MSD or TSQ-MS/MS