Fast, 3.5 Minute Analysis of Psilocin and Psilocybin in Urine by LC-MS/MS

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Abstract

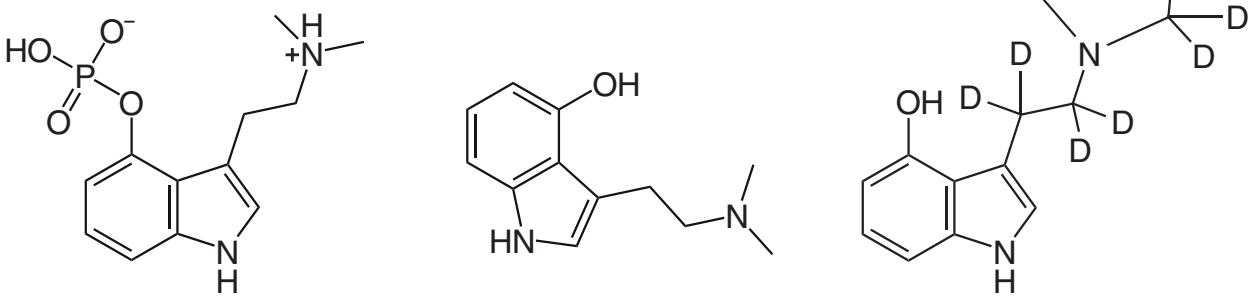
In this rapid LC-MS/MS analysis of psilocin and psilocybin in urine, supernatant from a simple protein precipitation was analyzed directly (no derivatization) on a Raptor Biphenyl column. Good quantitative results were obtained for linearity, accuracy, precision, and robustness in just 3.5 minutes. In addition, these compounds were analyzed using an established method for drugs of abuse/pain management medications, providing labs with an option to improve efficiency.

Introduction

Psilocybin-containing mushrooms, commonly called "magic mushrooms," contain two hallucinogenic indole alkaloids, psilocin and psilocybin, which possess mind-altering properties. While magic mushrooms are currently illegal in the U.S., psilocybin is a potential therapeutic for cluster headaches, anxiety, depression, and other disorders, so several municipalities have decriminalized microdosing of mushroom extracts. As therapeutic use is being explored, the need for routine diagnostic testing is growing. However, typical LC-MS/MS methods for analysis of psilocin and psilocybin can be problematic for several reasons. The primary chromatographic issue is that these alkaloids are highly polar and require a column with retention mechanisms beyond traditional hydrophobic interactions to adequately retain and separate them from matrix interferences. In addition, analysis is further complicated because psilocybin breaks down into psilocin via cleavage of the phosphate moiety (Figure 1) during in-source fragmentation.

Due to increased interest in the analysis of psilocin and psilocybin, we developed a simple and robust LC-MS/MS analytical method that pairs a quick solvent protein precipitation with a fast chromatographic separation. For this work, a Raptor Biphenyl column was selected because the phenyl-based stationary phase has been shown to provide good retention of hydrophilic compounds that elute early on alkyl (C18) phases due to enhanced pi-pi interactions. Here, in addition to evaluating method performance, we demonstrated that both compounds could be successfully added to an established method for 231 drugs of abuse [1]. Adding psilocin and psilocybin to an existing drug panel method instead of setting up an independent method can allow labs to operate much more efficiently.

Figure 1: Chemical Structures



Psilocybin **Psilocin** **Psilocin-D10**

Experimental

Calibration Standards and Quality Control Samples

First, 10 individual lots of human urine (BioreclamationIVT) were screened to confirm that both psilocin and psilocybin were not present. Then, the lots were pooled and fortified with psilocin and psilocybin to prepare calibration and QC standards. The linear range of the calibration curve was 50-5000 ng/mL, and four QC levels were prepared at 50 (LLOQ), 125 (LQC), 700 (MQC), and 4000 (HQC) ng/mL, respectively. For quantitation, psilocin D-10 (200 ng/mL) was used as the internal standard.

Sample Preparation

A 500 ng/mL sample of psilocin and psilocybin was prepared in pooled urine. A 50 µL aliquot was taken from the spiked urine sample and mixed with 10 µL of internal standard (psilocin-D10, 20 µg/mL) and 100 µL of methanol. The mixture was then vortexed at 3000 rpm for 10 seconds and centrifuged at 4300 rpm for 10 minutes at 10 °C. After centrifugation, 100 µL of the supernatant was diluted with 900 µL (20-fold dilution) of mobile phase A (0.1% formic acid and 2 mM ammonium formate in water) and injected for LC-MS/MS analysis.

Chromatographic Method:

The chromatographic conditions used on a Sciex 4000 coupled with a Shimadzu Prominence HPLC for this LC-MS/MS analysis of psilocin and psilocybin in urine are detailed below. The ion transitions and internal standards used for each analyte are provided in Table I.

Column: Raptor Biphenyl 2.7 µm, 50 mm x 2.1 mm (cat.# 9309A52)

Guard column: Raptor Biphenyl EXP guard column cartridge 2.7 µm, 5 mm x 2.1 mm (cat.# 9309A0252)

Column temp.: 35 °C

Injection volume: 5 µL

Mobile phase A: 0.1% Formic acid, 2 mM ammonium formate in water

Mobile phase B: 0.1% Formic acid, 2 mM ammonium formate in methanol

Time (min)	%A	%B
0.00	95	5
0.20	95	5
2.50	5	95
2.51	95	5
3.50	95	5

Flow rate: 0.5 mL/min

Ion mode: Positive ESI

Table I: Ion Transitions for LC-MS/MS Analysis of Psilocin and Psilocybin in Urine

Peak Identification	Precursor Ion	Product Ion Quantifier	Product Ion Qualifier
Psilocybin	285.1	205.1	240.0
Psilocin	205.1	160.1	115.0
Psilocin-D10 (IS)	215.1	66.1	-

Results and Discussion

Chromatographic Performance and Robustness

In this analysis of psilocin and psilocybin, both compounds were sufficiently retained on the Raptor Biphenyl column, and good baseline resolution was achieved in a fast, 3.5-minute total cycle time (Figure 2). In addition, the assay was free of interference from the urine matrix and other ions (Figures 2 and 3). Carryover was assessed by injecting a blank sample directly after injection of the HLOQ sample, and no carryover was observed. As shown in Figure 4, good robustness was demonstrated by the consistent peak shapes and retention times that were still observed after 500 injections of a pooled urine extract fortified at 500 ng/mL. The maximum system pressure also remained consistent indicating that no column clogging had occurred.

Linearity, Accuracy, and Precision

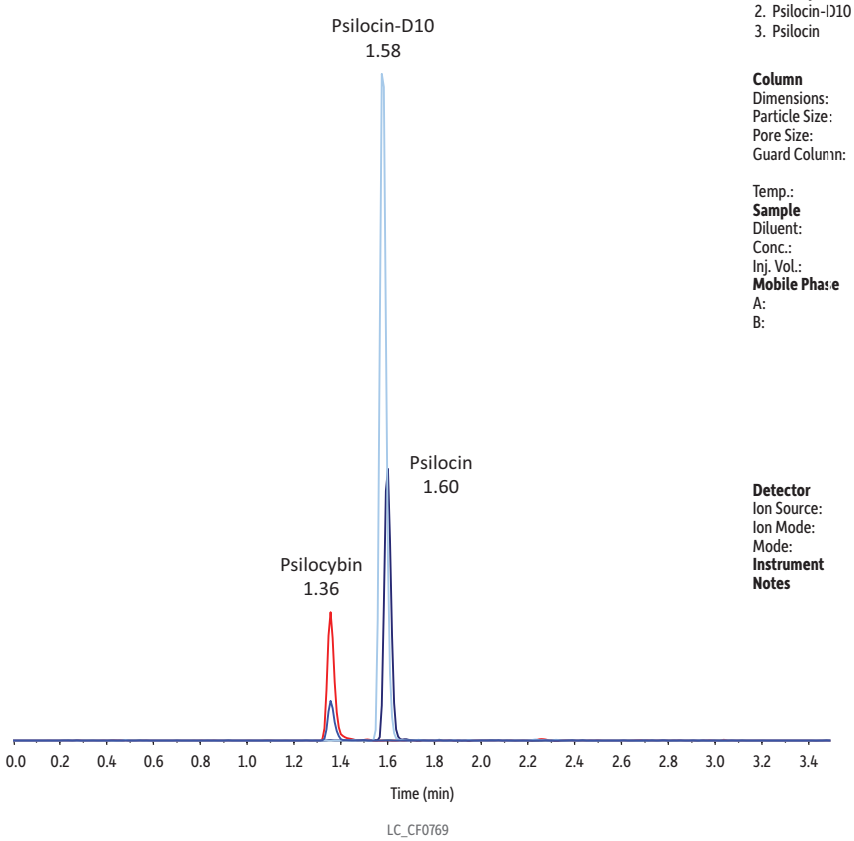
Using a 1/x weighted linear regression for both analytes, psilocin and psilocybin showed acceptable linearity with r^2 values of 0.995 or greater (Figure 5). In addition, the signal-to-noise values of the lowest calibration standards ranged from 16 to 65, indicating that this method could be used for the detection of much lower concentrations, if needed.

Precision and accuracy analysis of QC samples was performed on three different days. Method accuracy was demonstrated by percent recovery values being within 20% of the nominal concentration for all QC levels on all three days. The %RSD was 1.07-16.0% and 0.2-14.2% for intraday and interday evaluations, respectively, indicating acceptable method precision was achieved (Table II).

Easy Addition of Psilocin and Psilocybin to Existing Methods

In addition to developing a standalone method, we also explored adding both psilocin and psilocybin to an existing method to avoid the need for additional instrumentation and time for this analysis alone. As demonstrated in Figure 6, both psilocin and psilocybin in urine were run using the exact conditions of our previously published pain panel method that accommodates the analysis of 231 analytes [1]. Both the analytes were well separated from each other with a retention factor >5. In addition, no compounds from the pain panel method with the same masses eluted prior to 6 minutes, ensuring good separation from the earlier eluting psilocin and psilocybin. However, if coelution did occur under different conditions, psilocin and psilocybin could still be specifically detected by MS/MS based on their Q1 and Q3 masses. The selectivity of the Raptor Biphenyl column allowed these new compounds to be added to an existing method, providing labs with an important technique for improving efficiency and productivity.

Figure 2: Analysis of Psilocin and Psilocybin in Fortified Human Urine (500 ng/mL)



Peaks	t_R (min)	Conc. (ng/mL)	Precursor Ion	Product Ion 1	Product Ion 2
1. Psilocybin	1.36	500	285.1	205.1	240.0
2. Psilocin-D10	1.58	200	215.1	66.1	-
3. Psilocin	1.60	500	205.1	160.1	115.0

Column
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Guard Column: Raptor Biphenyl EXP guard column cartridge 5.0 mm, 2.1 mm ID, 2.7 µm (cat.# 9309A0252)
Temp.: 35 °C

Sample
Diluent: Water, 0.1% formic acid + 2 mM ammonium formate
Conc.: 500 ng/mL
Inj. Vol.: 5 µL

Mobile Phase
A: Water, 0.1% formic acid + 2 mM ammonium formate
B: Methanol, 0.1% formic acid + 2 mM ammonium formate

Time (min)	Flow (mL/min)	%A	%B
0.00	0.5	95	5
0.20	0.5	95	5
2.50	0.5	5	95
2.51	0.5	95	5
3.50	0.5	95	5

Detector MS/MS
Ion Source: Electrospray
Ion Mode: ESI+
Mode: MRM
Instrument HPLC
Notes A 500 ng/mL standard mix of psilocin and psilocybin was prepared in pooled urine. A 50 µL aliquot was taken from the standard and mixed with 10 µL of internal standard (psilocin-D10, 20 µg/mL) and 100 µL of methanol. The mixture was vortexed at 3000 rpm for 10 seconds and then centrifuged at 4300 rpm for 10 minutes at 10 °C. After centrifugation, 100 µL of the supernatant was diluted with 900 µL (20-fold dilution) of water containing 0.1% formic acid and 2 mM ammonium formate (mobile phase A) and injected for LC-MS/MS analysis.

Figure 3: Blank Human Urine Spiked with Internal Standard Only

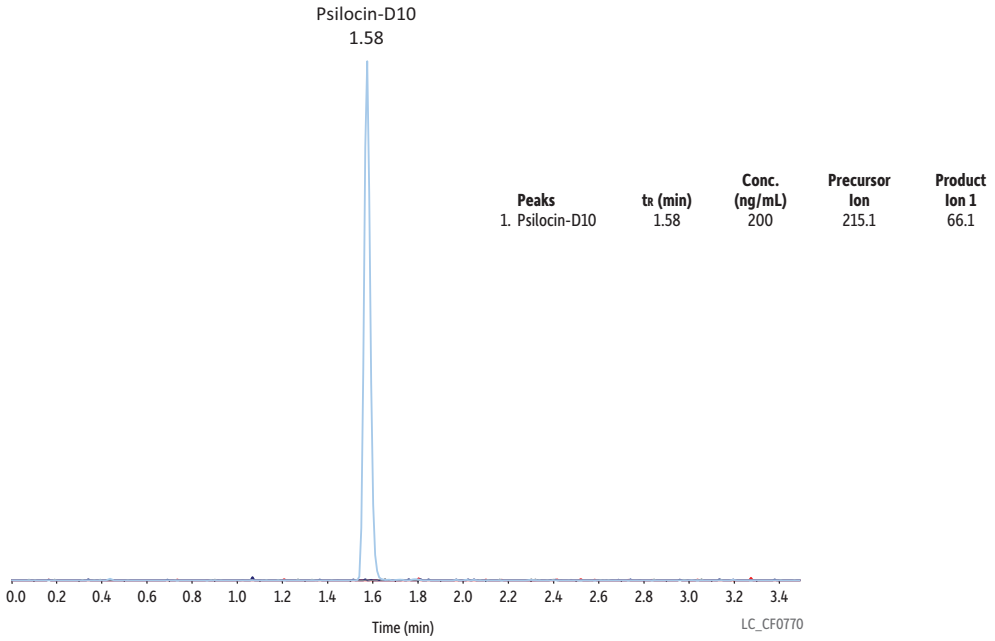


Figure 4: Robust column performance over 500 urine sample injections was obtained on a Raptor Biphenyl column. Good retention, consistent peak shapes, and stable retention times were seen over the course of the experiment.

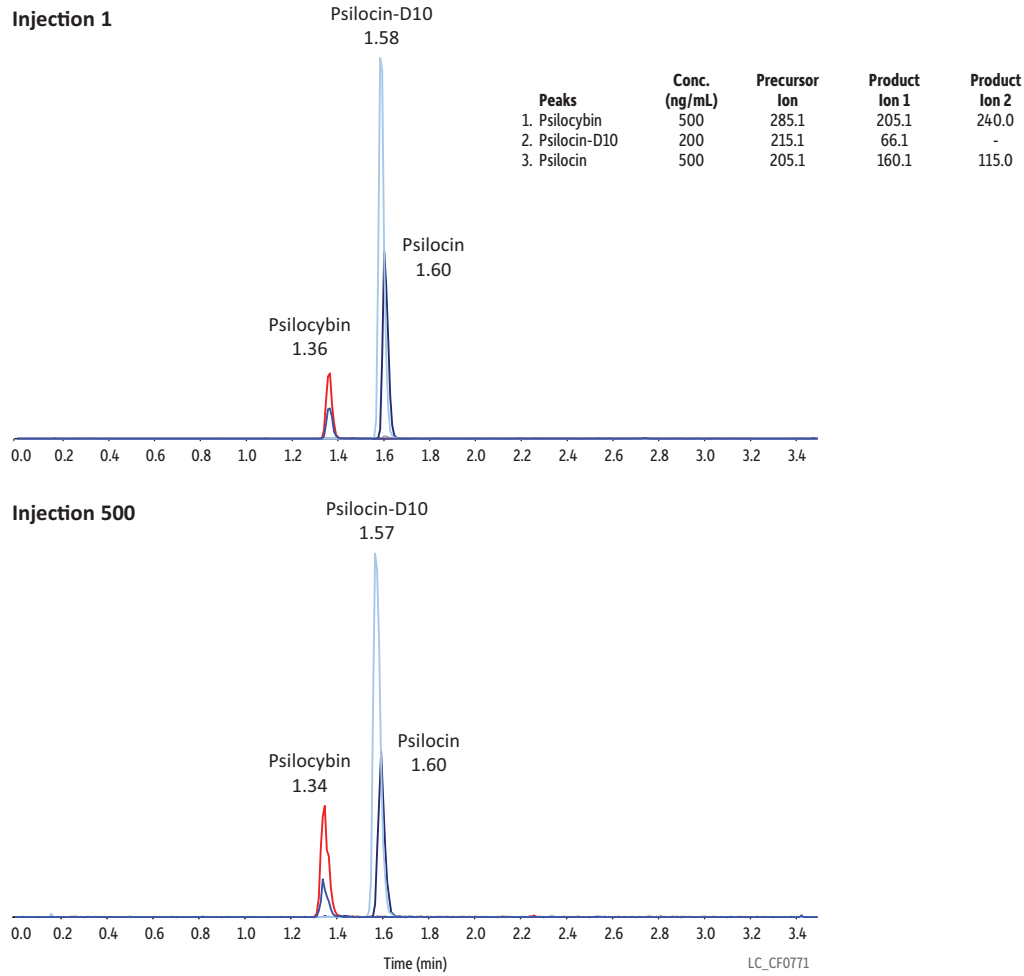


Figure 5: Standard Curves for the Analysis of Psilocin and Psilocybin

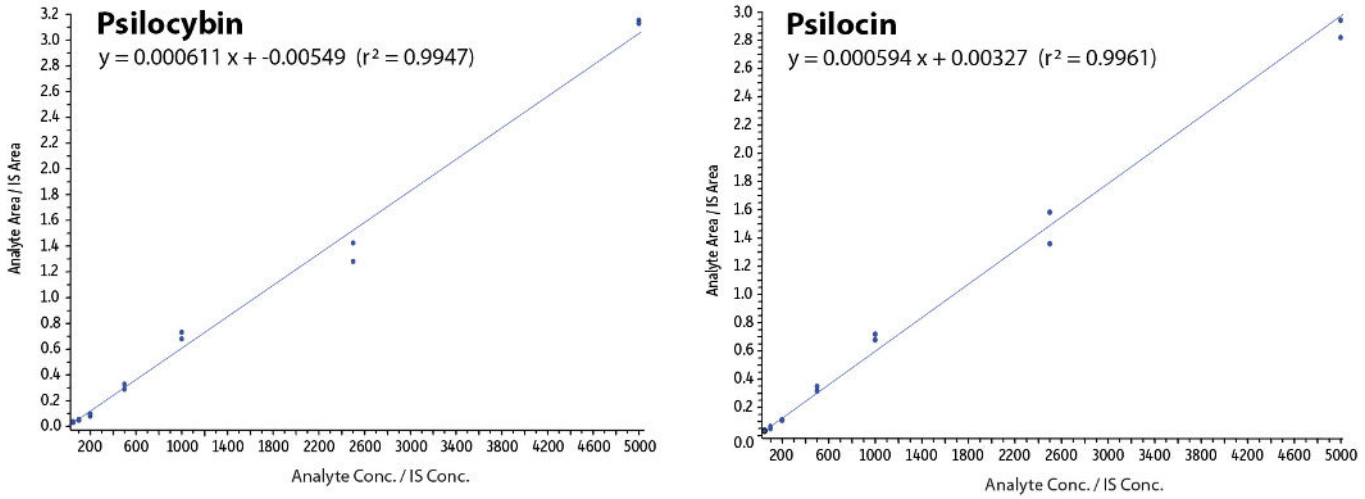
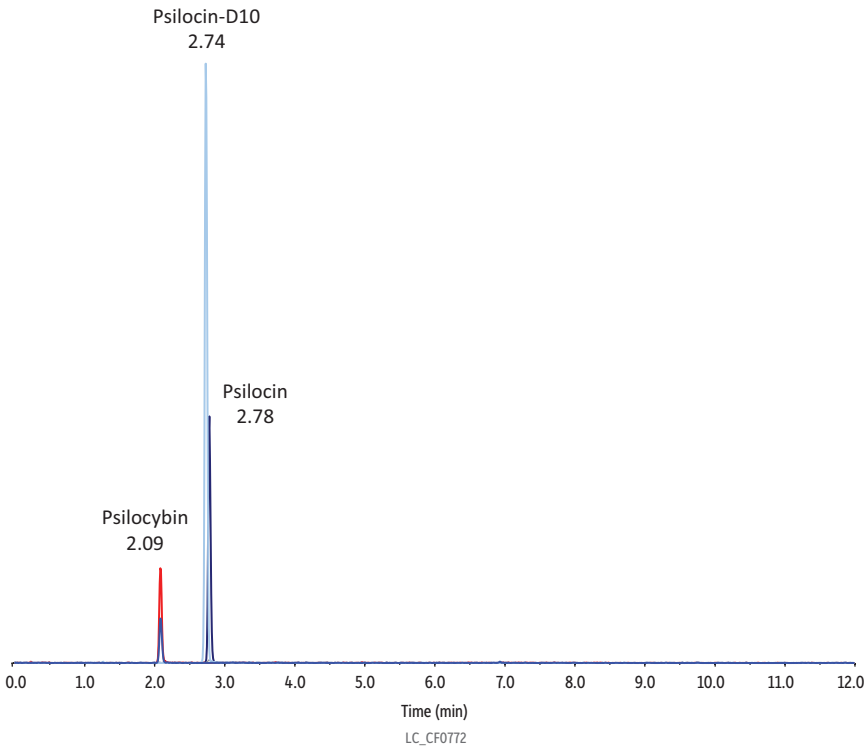


Table II: Accuracy and Precision of QC Samples

Analyte	QC LLOQ (50 ng/mL)			QC Low (125 ng/mL)			QC Mid (700 ng/mL)			QC High (4000 ng/mL)		
	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	% RSD	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	% RSD	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	% RSD	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	% RSD
Psilocybin	57.4	115	3.90	111	88.8	14.2	620	88.5	0.200	3730	93.3	7.10
Psilocin	55.7	112	8.30	147	118	3.90	655	93.5	3.80	4200	106	2.30

Figure 6: Analysis of psilocin and psilocybin in urine using the existing "Big Pain" multi-analyte method.



Peaks	t_R (min)	Conc. (ng/mL)	Precursor Ion	Product Ion 1	Product Ion 2
1. Psilocybin	2.09	500	285.1	205.1	240.0
2. Psilocin-D10	2.74	200	215.1	66.1	-
3. Psilocin	2.78	500	205.1	160.1	115.0

Column Raptor Biphenyl (cat.# 9309A12)
Dimensions: 100 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Guard Column: Raptor Biphenyl EXP guard column cartridge 5.0 mm, 2.1 mm ID, 2.7 µm (cat.# 9309A0252)
Temp.: 30 °C

Sample
Diluent: Water, 0.1% formic acid + 2 mM ammonium formate
Conc.: 500 ng/mL
Inj. Vol.: 5 µL

Mobile Phase
A: Water, 0.1% formic acid + 2 mM ammonium formate
B: Methanol, 0.1% formic acid + 2 mM ammonium formate

Time (min)	Flow (mL/min)	%A	%B
0.00	0.6	95	5
9.00	0.6	0	100
10.00	0.6	0	100
10.01	0.6	95	5
12.00	0.6	95	5

Detector MS/MS
Ion Source: Electrospray
Ion Mode: ESI+
Mode: MRM
Instrument HPLC
Notes A 500 ng/mL standard mix of psilocin and psilocybin was prepared in pooled urine. A 50 µL aliquot was taken from the standard and mixed with 10 µL of internal standard (psilocin-D10, 20 µg/mL) and 100 µL of methanol. The mixture was vortexed at 3000 rpm for 10 seconds and centrifuged at 4300 rpm for 10 minutes at 10 °C. After centrifugation, 100 µL of the supernatant was diluted with 900 µL (20-fold dilution) of water containing 0.1% formic acid and 2 mM ammonium formate (mobile phase A) and injected for LC-MS/MS analysis.

Conclusion

A fast, 3.5-minute chromatographic separation employing a simple protein precipitation and a Raptor Biphenyl column was developed for the quantitative analysis of psilocin and psilocybin in human urine. The use of a Raptor Biphenyl column ensured adequate retention and provided baseline separation, consistent peak shapes, and stable retention times over the course of 500 matrix injections. In addition to this robust method, these compounds can be analyzed by an established method for drugs of abuse/pain management medications, which affords labs an important opportunity to increase productivity and efficiency.

References

S. Lupo, "The Big Pain": Development of Pain-Free Methods for Analyzing 231 Multiclass Drugs and Metabolites by LC-MS/MS, Restek Corporation, 2016 https://www.restek.com/technical-literature-library/articles/the-big-pain-development-of-pain-free-methods-for-analyzing-231-multiclass-drugs-and-metabolites-by-LC-MSMS

Raptor Biphenyl LC Columns (USP L11)

- Ideal for bioanalytical testing applications like drug and metabolite analyses.
- Heightened selectivity and retention for compounds that are hard to resolve or elute early on C18 and other phenyl chemistries.
- Limits ionization suppression and allows simple, MS-friendly mobile phases.
- Part of Restek's Raptor LC column line featuring 1.8, 2.7, and 5 µm SPP core-shell silica.

ID	Length	qty.	cat.#
2.7 µm Particles			
2.1 mm	50 mm	ea.	9309A52
	100 mm	ea.	9309A12

ordering notes

Certificates of analysis for new Restek LC columns are now provided electronically. To view and download, visit www.restek.com/documentation then enter your cat.# and serial #.

Raptor EXP Guard Column Cartridges

- Free-Turn architecture lets you change cartridges by hand without breaking inlet/outlet fluid connections—no tools needed.
- Patented titanium hybrid ferrules can be installed repeatedly without compromising high-pressure seal.
- Auto-adjusting design provides ZDV (zero dead volume) connection to any 10-32 female port.
- Guard column cartridges require EXP direct connect holder (cat.# 25808).
- Pair with EXP hand-tight fitting (cat.# 25937–25938) for tool-free installation.

Description	Particle Size	Size	qty.	cat.#
Raptor Biphenyl EXP Guard Column Cartridge	2.7 µm	5 x 2.1 mm	3-pk.	9309A0252

Maximum cartridge pressure: 1034 bar/15,000 psi* (UHPLC), 600 bar/8700 psi (2.7 µm); 400 bar/5800 psi (5 µm)
* For maximum lifetime, recommended maximum pressure for UHPLC particles is 830 bar/12,000 psi.
Intellectual Property: optimizetech.com/patents

25808

EXP Direct Connect Holder

Description	qty.	cat.#
EXP Direct Connect Holder for EXP Guard Cartridges (includes hex-head fitting & 2 ferrules)	ea.	25808

Maximum holder pressure: 20,000 psi (1400 bar)
Intellectual Property: optimizetech.com/patents

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