WHITE PAPER

Pain Management Panel – Getting Accurate Results No Matter the Matrix

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Introduction

Pain management drugs are very common in the drug market. However, the ability to quantify and determine the presence of minute amounts of pain management drugs in various and complex sample matrices remains an issue due to the extensive and time-consuming sample preparation that is required. Previously, these drugs were measured using immunoassays such as enzyme-linked immunosorbent assay (ELISA), but there is a high frequency of interference from the sample matrix, along with a lack of specificity. Advancements in both the chromatography and sample clean-up have made it less difficult to develop accurate methods and also has allowed labs to process more samples in less time to keep up with the market demands. LC-MS/MS provides a highly sensitive and specific approach to determining the concentrations of structurally related molecules and offers a robust platform with high sensitivity and specificity for measuring pain management drugs simultaneously. Sample preparation is key in order to minimize potential interference from the sample matrix. It also aids in preventing an HPLC column from clogging or premature column failure due to unwanted extractables. Solid Phase Extraction (SPE) is an effective technique for cleaning up and concentrating samples. The SPE sample needs to meet certain conditions to allow for reproducible, highly efficient solid phase extraction. First, the sample should be a liquid of low viscosity. This allows for easy passage through the SPE cartridge. Second, the sample should have a low content of solids or particulate contaminants to prevent clogging of the cartridge and, ultimately, the HPLC column. Finally, the solvent composition needs to be suitable for retention.

With an increasing number of clinical research applications being developed from bioanalytical samples, the removal of phospholipids from the sample prior to HPLC analysis has become an important step for accurate detection. Phospholipids are present in a majority of bioanalytical samples including whole blood and plasma. When injected, phospholipids have been shown to reduce MS sensitivity owing to ion suppression. Phree™ phospholipid removal (PLR) solutions offer a fast and effective way to remove both proteins and phospholipids in a single product platform without negatively affecting the recovery of your target analytes.

In this white paper, we examine three different matrices and the techniques that can be used to clean samples prior to running them on HPLC for the detection of a panel of 39 pain management drugs (**Table 1**).

Table 1. 39 Pain Management Analytes

Peak ID	Analyte Name	Peak ID	Analyte Name
1	Alprazolam	21	Flurazepam
2	Amphetamine	22	Hydrocodone
3	Benzoylecgonine	23	Hydromorphone
4	Codeine	24	Lorazepam
5	Diazepam	25	MDA
6	MDMA	26	MDEA
7	Methamphetamine	27	Meperidine
8	Norbuprenorphine	28	Methadone
9	Oxazepam	29	Midazolam
10	Oxymorphone	30	Morphine
11	PCP	31	Naloxone
12	Propoxyphene	32	Naltrexone
13	Sufentanil	33	Nordiazepam
14	6MAM	34	Norfentanyl
15	Buprenorphine	35	Normeperidine
16	Carisoprodol	36	Norpropoxyphene
17	Clonazepam	37	Oxycodone
18	EDDP	38	Temazepam
19	Fentanyl	39	Tramadol
20	Flunitrazepam		

Urine

Many of these panel drugs in urine matrix are present in conjugated metabolized form, that needs to undergo hydrolysis in the sample pre-treatment prior to extraction. Solid Phase Extraction (SPE) is a sample prep tool that will not just effectively remove matrix interferences but also the enzyme used in the hydrolysis step. Enzymes that are essentially proteins are susceptible to protein precipitation and can clog a LC column due to exposure to a higher percentage of organic during the gradient of the mobile phases in a chromatographic run. Strata™X-Drug B Plus Solid Phase Extraction (SPE) features an easy clean up solution by eliminating a few steps in a SPE extraction. It uses an in-well beta-glucuronidase hydrolysis to save time and transfer steps. This SPE method does not require conditioning or equilibration steps for additional time savings. When combined with a core-shell Kinetex Biphenyl LC column, absolute recovery yields are between 71-112%, with good separation of analytes and isomeric compounds are observed.

Sample Preparation Protocol

Sample Pre-treatment: Combine 200 µL urine sample spiked with 40 µL internal standard (500 ng/mL), 60 µL hydrolysis buffer, 20 µL of IMCSzyme® RT enzyme (Part No.: 04-RTB-030), on the Strata-X-Drug B Plus, 30 mg plate (Part No.: 8E-S128-TGB-P). Incubate at room temperature for 15 minutes

Load (by applying vacuum): Add 200 µL 0.1 % Formic acid in Water to the plate, mix/vortex for a minute followed by application of vacuum to absorb the sample on the SPE media

Wash 1: 1 mL of 0.1 % Formic acid in Water

Wash 2: 1 mL of Water/Methanol (70:30)

Dry: 5 minutes at high vacuum (15-20" Hg)

Elute: 2x 0.5 mL Ethyl acetate/Isopropanol/Ammonium hydroxide (7:2:1)

Dry Down: Under gentle stream of Nitrogen at 40-45°C

Reconstitute: 200 µL initial mobile phase

LC Conditions 1 (Quantitative Analysis of Drug Research Panel)

Column: Kinetex™ 2.6 µm Biphenyl

Dimensions: 50 x 3.0 mm

Part Number: 00B-4622-Y0

Mobile Phase: A: 0.1% Formic acid in Water
B: 0.1% Formic acid in Methanol

Gradient:

Time (min)	% B
0	15
3.5	95
5	95
5.01	15
7	15

Flow Rate: 0.5 mL/min

Temperature: Ambient

Injection: 5 µL

Detection: SCIEX® 4500 MS/MS (ESI+)

Detector: Agilent® Technologies 1200 Series

Figure 1. % Recoveries and CVs for 39 Pain Management Drugs using an In-well Hydrolysis SPE Product **Figure 2** with linear velocity scaled to ID (i.e. 1 mL/min)

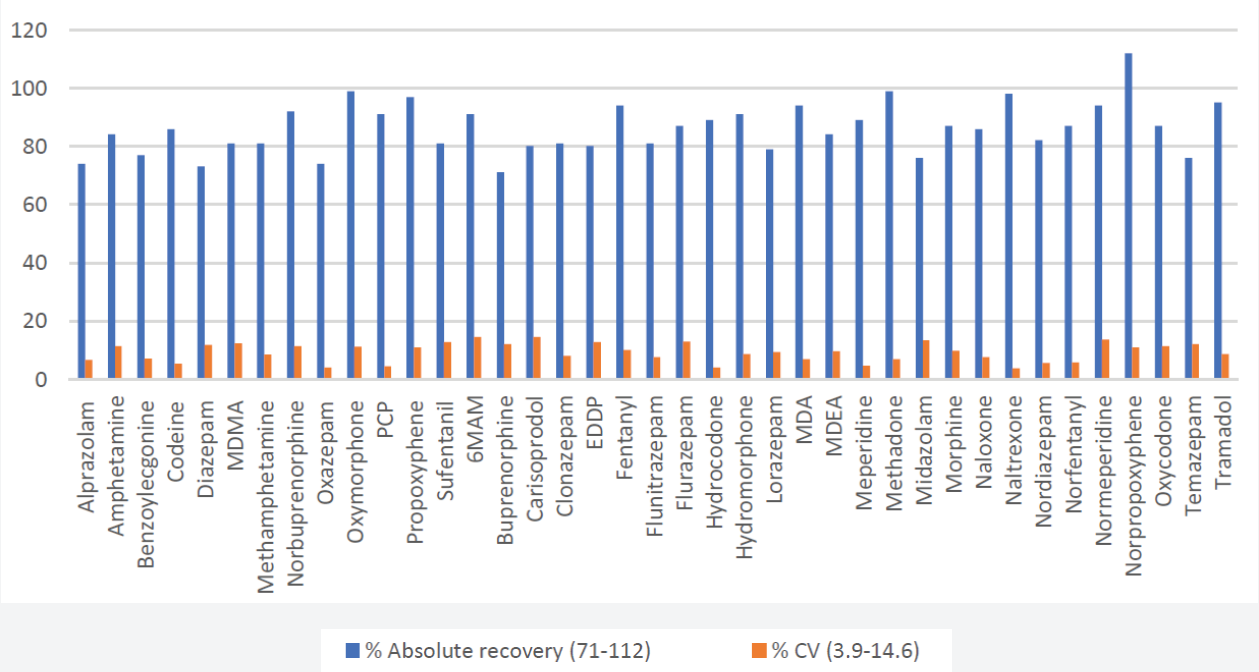
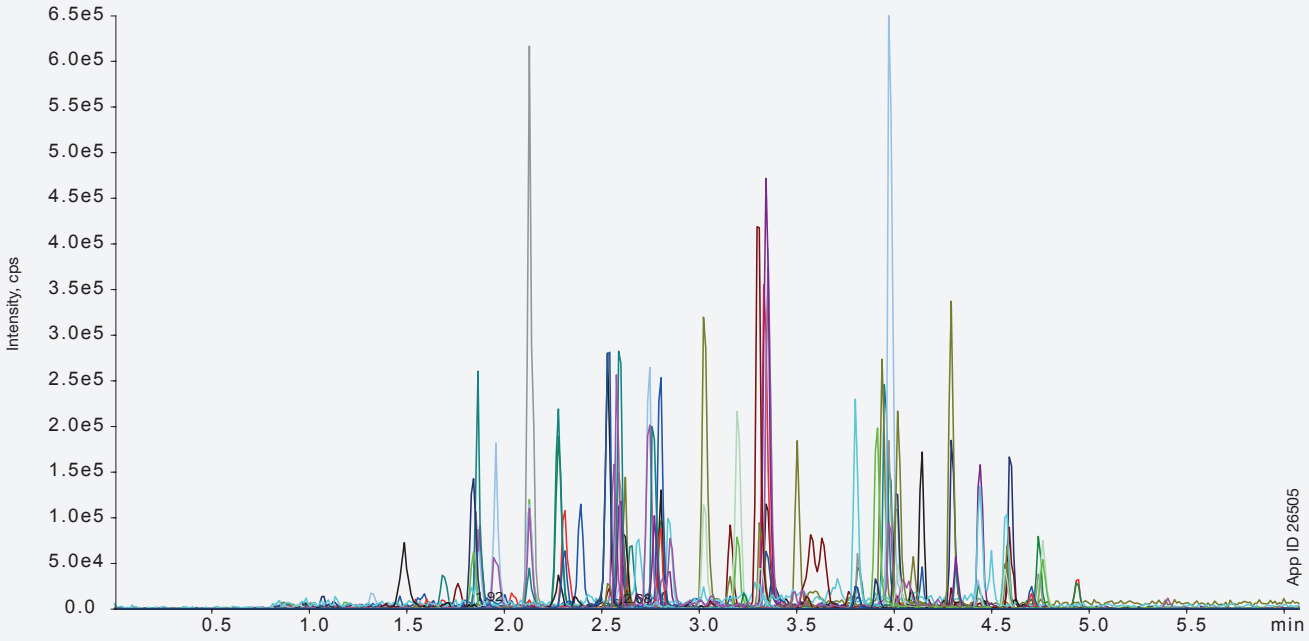


Figure 2. Separation of 39 Pain Panel Analytes using SPE with In-well Hydrolysis



Whole Blood

Whole blood is a common matrix for toxicology testing, yet it can be very complicated to clean up which is necessary before injection for analysis. Whole blood as a matrix does have disadvantages due to the complexity and the required pre-treatment steps even before the appropriate sample preparation. The whole blood components must be broken down and the clean-up solution required is a phospholipid removal or a solid phase extraction product. Using Phree™ Phospholipid Removal as a pass through with an easy protein precipitation, the whole blood matrix is efficiently cleaned up while still yielding high recoveries and low variation before analysis on LC-MS/MS using a Kinetex™ Biphenyl LC column. Using a phospholipid removal solution allows the phospholipids to be removed prior to MS analysis which, if not removed, could cause serious damage to the column or the MS instruments.

Sample Preparation Protocol

Sample Pretreatment: 200 µL of serum was aliquoted into a tube and 600 µL of chilled (0 to -20°C) Acetonitrile/Methanol (95:5) was added and vortex/mixed for 5-10 seconds. The tube was centrifuged at 3000 rpm for 10 minutes and the supernatant was collected and 25 µL of 1% formic acid was added.
Load: Pre-treated sample onto the Phree™ 96-well Plate (Part No.: 8E-S138-TGB)
Vacuum: 4-5 psi to collect supernatant
Dry Down: Sample under a gentle stream of Nitrogen at 40-45°C
Reconstitute: 200 µL initial mobile phase

Figure 3. Absolute Recoveries and % CVs for 39 Drugs Extracted from Whole Blood Using Phree PLR

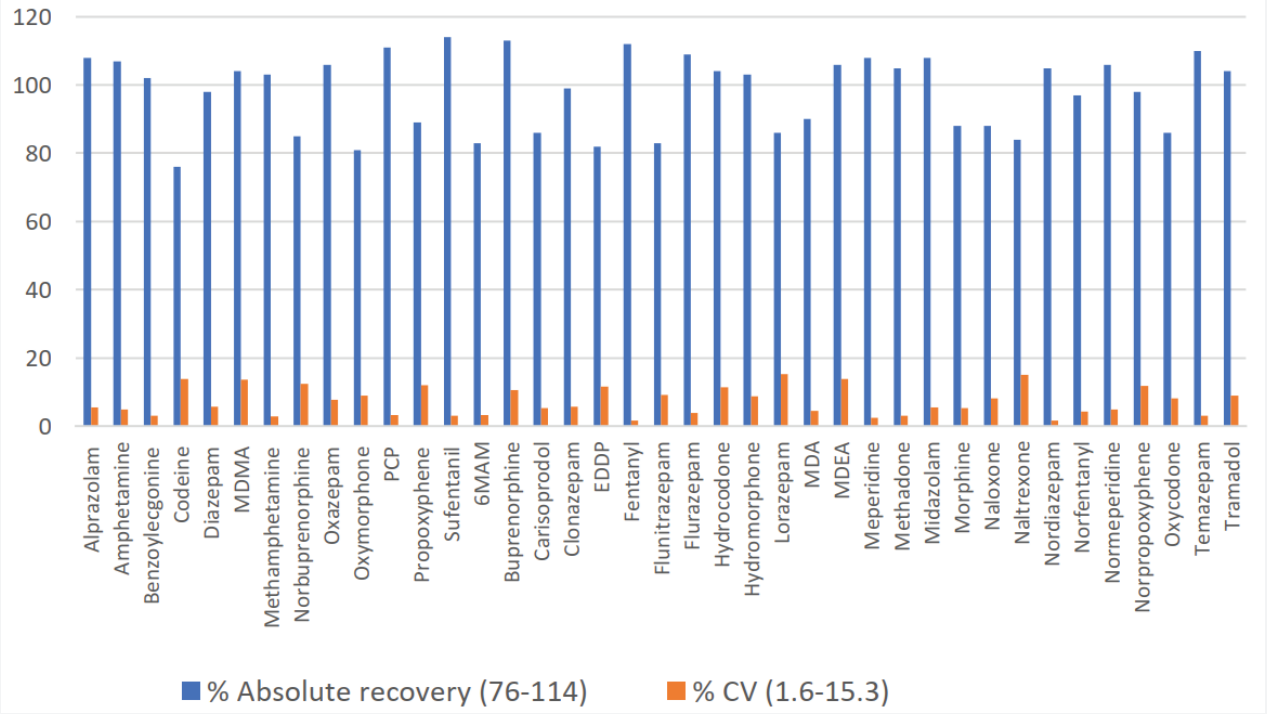
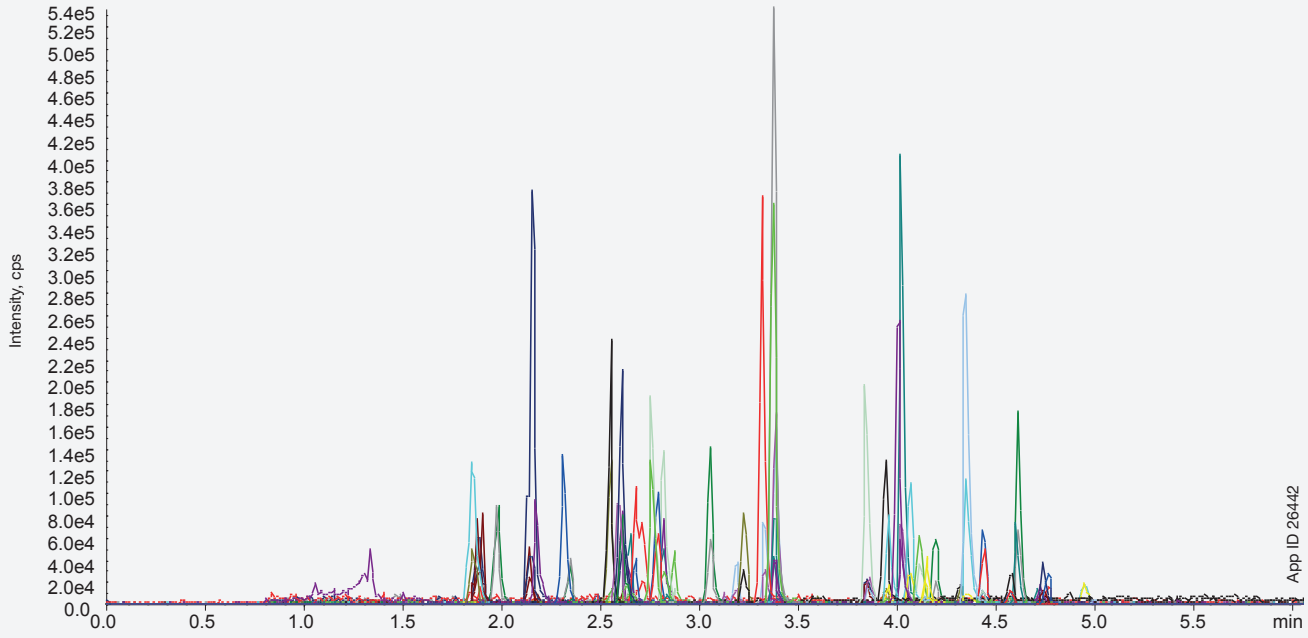


Figure 4. Separation of 39 Pain Panel Analytes from Whole Blood



LC Conditions 2 (Quantitative Analysis of of Phospholipids)

Column: Kinetex™ 2.6 µm C18
Dimensions: 50 x 2.1 mm
Part Number: 00B-4462-AN
Mobile Phase: A: 0.1% Formic acid in Water
B: 0.1% Formic acid in Methanol

Gradient:

Time (min)	% B
0	40
0.5	95
11.5	95
11.51	40
13.5	40

Flow Rate: 0.4 mL/min
Temperature: 40 °C
Injection: 5 µL
Detection: SCIEX® 4500 MS/MS (ESI+)
Detector: Agilent® Technologies 1200 Series

Figure 5a. Phospholipid Content from Whole Blood Extracted by Protein Precipitation

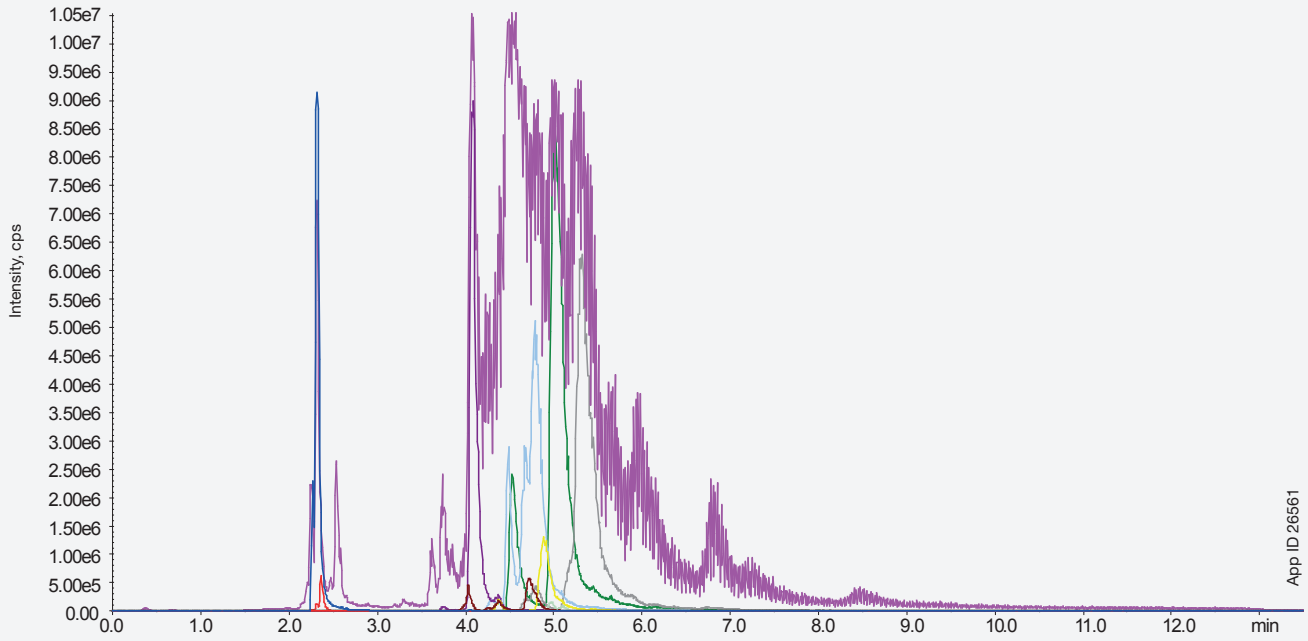
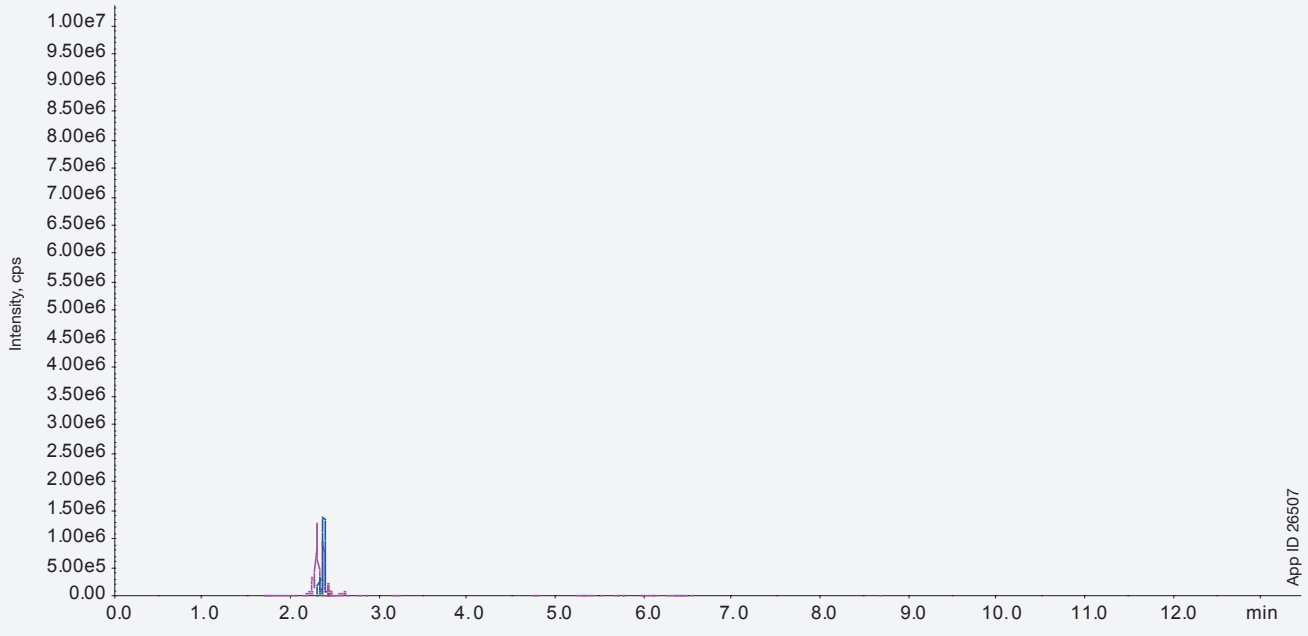


Figure 5b. Phospholipid Content from Whole Blood Extracted by Phree™ PLR



Serum

For analysis of drugs, plasma and serum allow for better real-time detection data. Plasma and serum act as a good indicator and can provide accurate LC results, but the sample is more complex and a simple dilution will not effectively clean up the sample for LC-MS/MS. This matrix needs either a protein precipitation, phospholipid removal, or SPE prior to analysis. Spending time developing an LC method with the correct selectivity and separation will be ineffective with the addition of proteins and phospholipids in the samples. The phospholipids present in the sample can cause ion suppression or enhancements in the MS system at specific transitions, further causing inaccurate results and a change in the baseline. Serum was cleaned up and compared with both a single protein precipitation protocol and also by the addition of a phospholipid removal product. The analysis was carried out with a Kinetex™ 2.6 µm Biphenyl LC column to show the separation similarities. The phospholipid traces show the differences between the two clean-up techniques and displays why Phree™ is a necessary addition to the clean-up steps.

Sample Preparation Protocol

Sample Pretreatment: 200 µL of serum was aliquoted into a tube and 600 µL of chilled (0 to -20°C) Acetonitrile/Methanol (95:5) was added and vortex/mixed for 5-10 seconds. The tube was centrifuged at 300 rpm for 10 minutes and the supernatant was collected and 25 µL of 1% formic acid was added.
Load: Pre-treated sample onto the Phree 96-Well Plate (Part No.: 8E-S133-TGB)
Vacuum: 4-5 psi to collect supernatant
Dry Down: Sample under a gentle stream of Nitrogen at 40-45 °C
Reconstitute: 200 µL initial mobile phase

Figure 6. Absolute Recoveries and % CVs for 39 Drugs Extracted from Serum Using Phree PLR

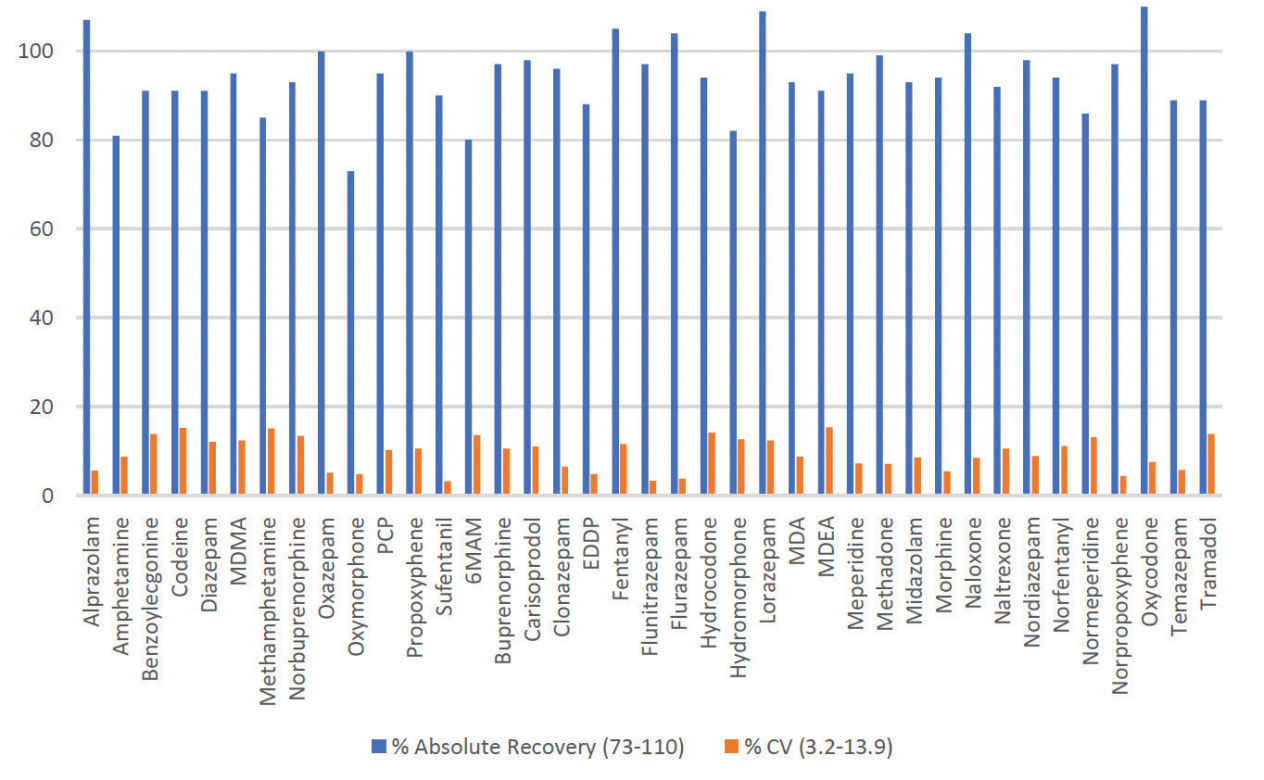


Figure 7. Phospholipid Content from Serum Extracted by Phree™ PLR

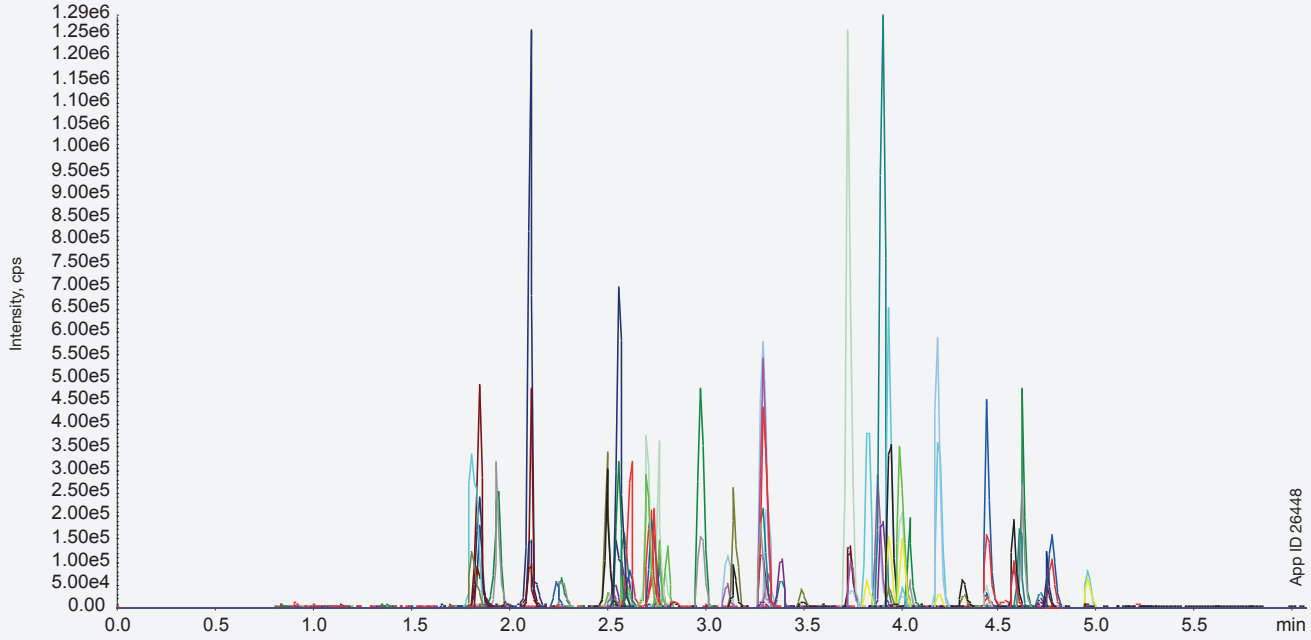


Figure 8. Separation of 39 Pain Panel Analytes using SPE with In-well Hydrolysis

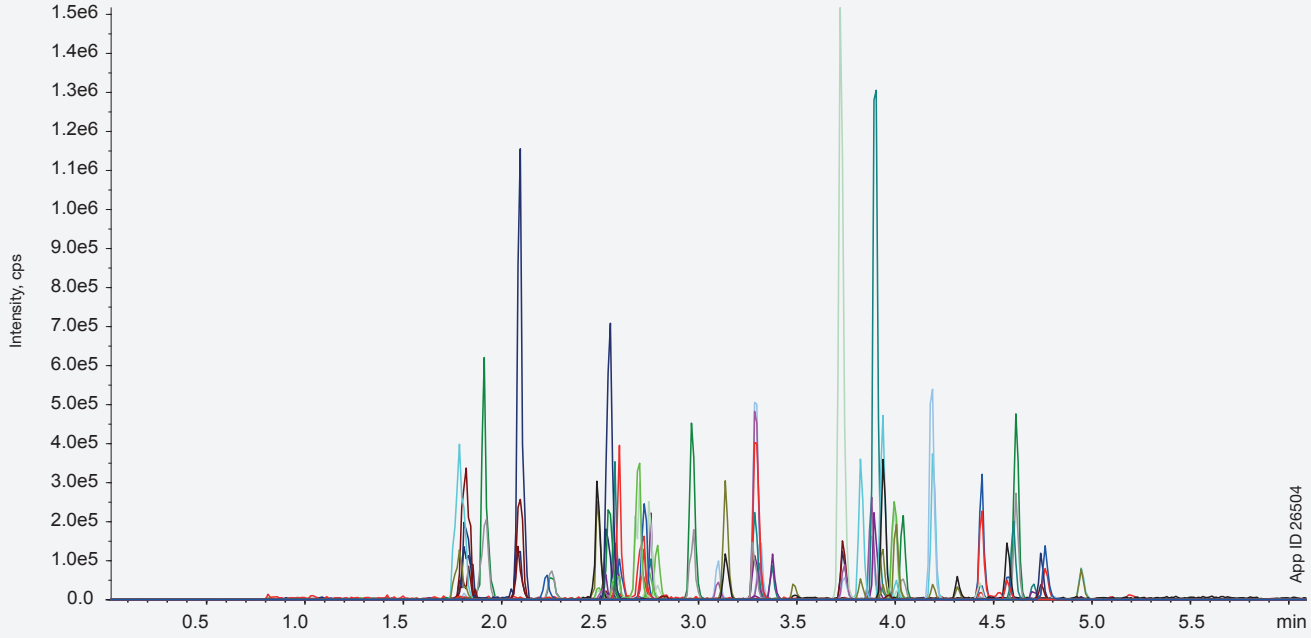


Figure 9a. Phospholipid Content from Serum Extracted by Protein Precipitation

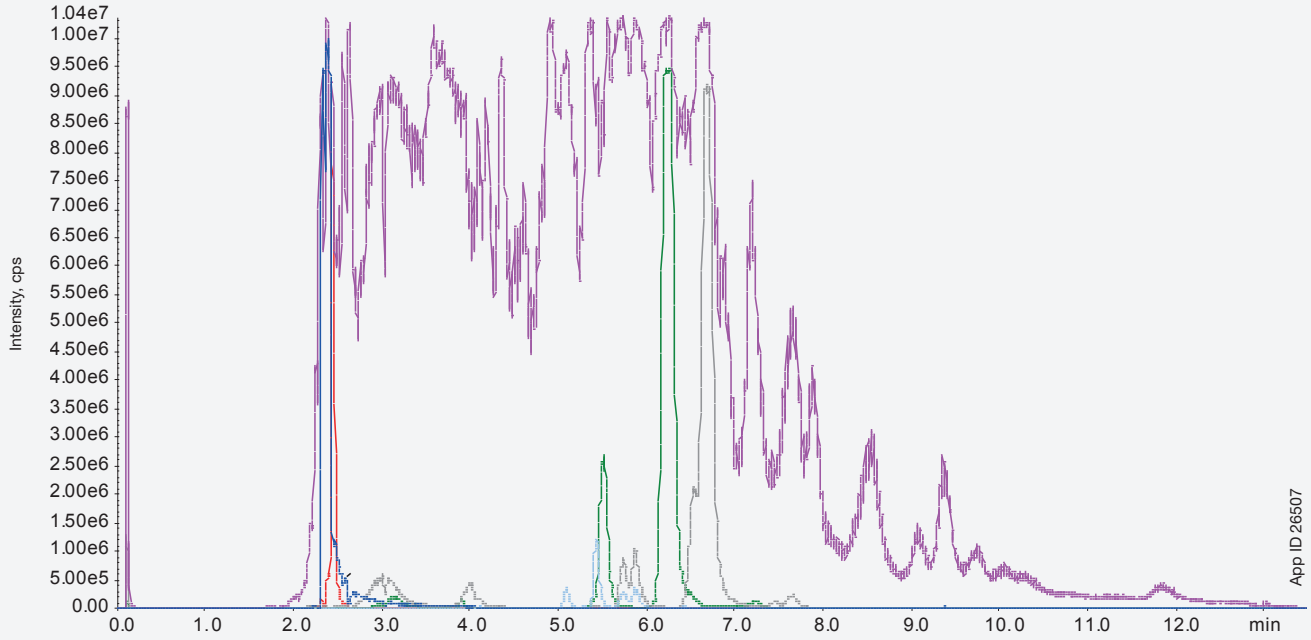


Figure 9b. Phospholipid Content from Serum Extracted by Phree™ PLR

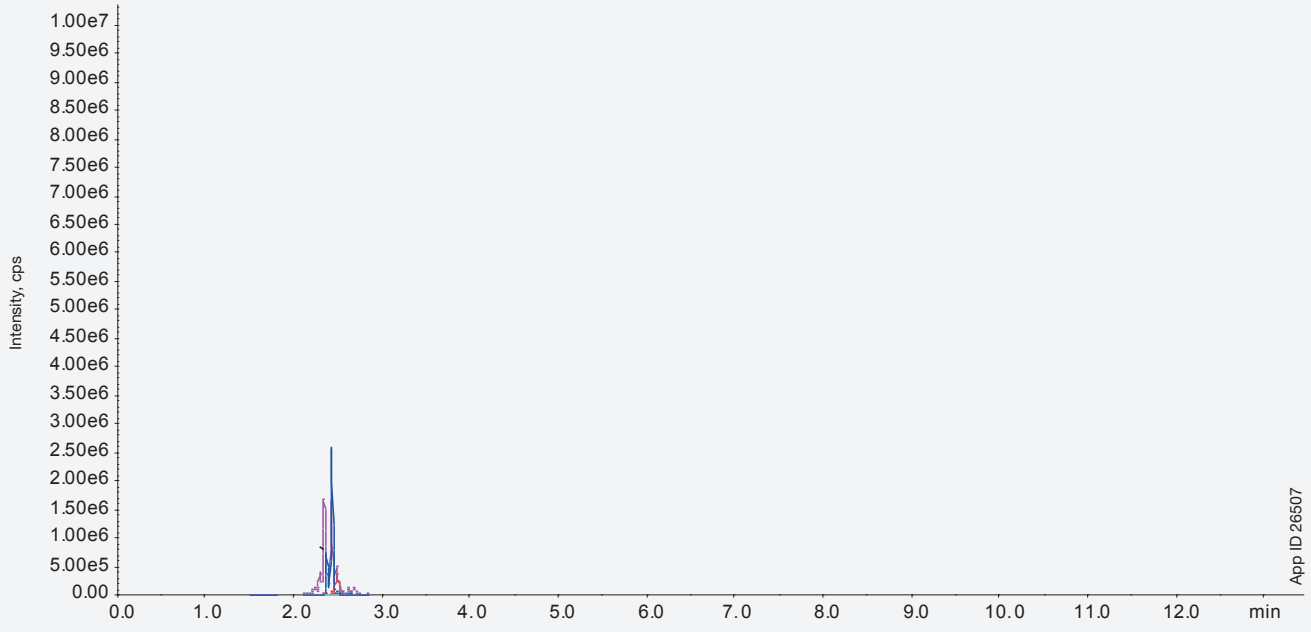
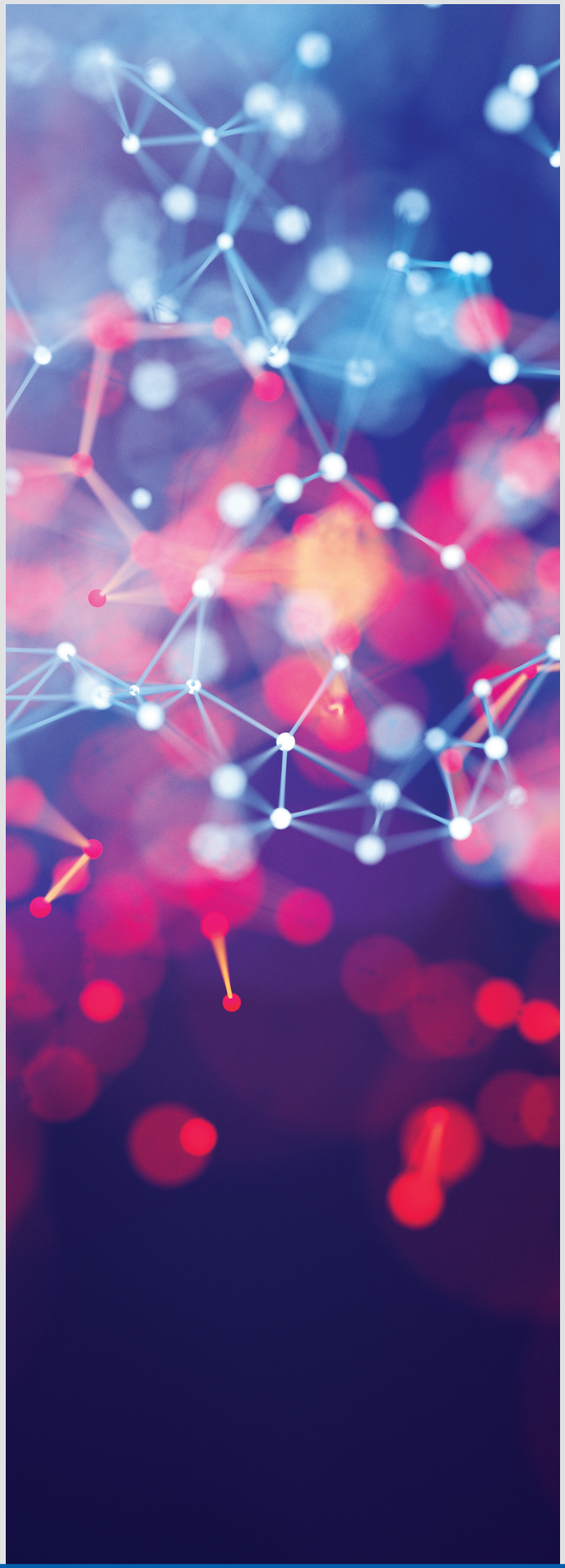


Table 2.

					Serum Phospholipid Removal		Whole Blood Phospholipid Removal		Urine SPE	
Peak No.	Analyte Name	RT (min)	Q1	Q3	% Rec.	% CV (N=4)	% Rec.	% CV (N=4)	% Rec.	%CV (N=4)
1	Alprazolam	4.8	309.1	281.1	107	5.6	108	5.5	74	6.7
2	Amphetamine	2.3	136.1	91.1	81	8.8	107	4.9	84	11.3
3	Benzoylecgonine	3.3	290.1	168.1	91	13.8	102	3.1	77	7.1
4	Codeine	2.6	300.2	152.1	91	15.2	76	13.9	86	5.4
5	Diazepam	4.9	285	193.2	91	12	98	5.7	73	11.9
6	MDMA	2.9	194.1	105.1	95	12.4	104	13.7	81	12.2
7	Methamphetamine	2.6	150.1	91	85	15	103	2.8	81	8.5
8	Norbuprenorphine	3.6	414.3	83.2	93	13.4	85	12.4	92	11.4
9	Oxazepam	4.4	287	241	100	5.1	106	7.8	74	4
10	Oxymorphone	2	302.1	227	73	4.8	81	8.9	99	11.1
11	PCP	4	244.3	91	95	10.3	111	3.3	91	4.4
12	Propoxyphene	4	340.3	266.3	100	10.6	89	12	97	11
13	Sufentanil	4.1	387.2	238.1	90	3.2	114	3.1	81	12.8
14	6MAM	2.57	328.1	165.1	80	13.5	83	3.3	91	14.6
15	Buprenorphine	3.9	468.3	55.2	97	10.6	113	10.5	71	12
16	Carisoprodol	3.9	261.1	176.2	98	11	86	5.3	80	14.5
17	Clonazepam	4.4	316.1	270.1	96	6.5	99	5.7	81	8
18	EDDP	4.2	278.2	234.2	88	4.8	82	11.6	80	12.8
19	Fentanyl	3.9	337.3	105.1	105	11.6	112	1.6	94	10.1
20	Flunitrazepam	4.7	314.1	268.2	97	3.3	83	9.1	81	7.6
21	Flurazepam	4	388.2	315.2	104	3.8	109	3.9	87	13
22	Hydrocodone	2.8	300.2	199	94	14.2	104	11.3	89	3.9
23	Hydromorphone	2.1	286.1	185.1	82	12.6	103	8.7	91	8.6
24	Lorazepam	4.3	321	275	109	12.3	86	15.3	79	9.3
25	MDA	2.7	180.1	133	93	8.8	90	4.4	94	7
26	MDEA	3	208.2	163	91	15.3	106	13.8	84	9.6
27	Meperidine	3.4	248.2	220.2	95	7.2	108	2.5	89	4.6
28	Methadone	4.4	310	265	99	7.1	105	3.1	99	6.9
29	Midazolam	4.1	326.1	291.1	93	8.6	108	5.5	76	13.4
30	Morphine	1.9	286.1	152.1	94	5.4	88	5.2	87	9.8
31	Naloxone	2.56	328.2	212	104	8.5	88	8.2	86	7.6
32	Naltrexone	2.8	342.2	267.1	92	10.5	84	15	98	3.7
33	Nordiazepam	4.64	271	140	98	8.9	105	1.6	82	5.5
34	Norfentanyl	3.2	233.2	84.1	94	11.2	97	4.3	87	5.7
35	Normeperidine	3.4	234.1	160.1	86	13.1	106	4.9	94	13.7
36	Norpropoxyphene	4.1	308.2	100.1	97	4.4	98	11.7	112	10.9
37	Oxycodone	2.8	316.1	241.2	110	7.6	86	8.2	87	11.4
38	Temazepam	4.7	301.1	255.1	89	5.8	110	3.1	76	12
39	Tramadol	3.2	264.1	58.1	89	13.9	104	9	95	8.7
	% Recovery range for 39 analytes				73-110%		76-114%		71-99%	

Conclusion

Phree™ PLR product combines the simplicity of protein precipitation and the selectivity of Solid Phase Extraction (SPE) providing selective elimination of majority of phospholipids while quantitatively and efficiently eluting the analyte of interest. The fast and effective sample clean up (in 96-well plate format) meets the demand of a high-throughput environment. Strata™-X Drug B Plus SPE for in-well hydrolysis accelerates sample processing time and greatly reduces the need for additional equipment while simultaneously improving both contamination and analyte loss issues. The prescribed solution can avoid column plugging and premature demise of the LC column, resulting in a cost effective, faster workflow.



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