Removal of Lipids for the Analysis of Toxicological Compounds in Plasma by LC/MS/MS

Enhanced Matrix Removal—Lipid Dispersive Cleanup

Application Note

Small Molecule, Toxicology

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Abstract

A convenient analytical method for the determination of toxicological compounds in plasma involves the addition of acetonitrile to a small volume of plasma. The mixture is vortexed to elicit protein precipitation, and centrifuged. The supernatant is transferred to a dispersive tube containing Enhanced Matrix Removal—Lipid (EMR—Lipid) sorbent to remove >97% endogenous plasma lipid matrix components. EMR—Lipid removed substantially more lipids than other phospholipid removing sorbents tested. Analytes are isolated from spiked plasma samples with accuracies above 95% and Relative Standard Deviations (RSDs) of <6% on average. Combining protein precipitation with EMR—Lipid dispersive SPE in plasma offered separation for various toxicological compounds, Limits of Quantification (LOQs) at 1 ng/mL or below based on method performance. The method is quick, easy, and removes lipids that are known to remain on the column causing chromatographic anomalies and source contamination.

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Introduction

Determination of toxicological compounds in biological matrices is commonly employed in clinical research. The main techniques used for analysis are immunoassays, LC, and GC. Mass spectral chromatographic methods are the first choice for many applications based on their flexibility, selectivity, sensitivity, qualitative, and quantitative capabilities. Analysis of toxicological compounds in biological samples requires sample preparation that can range from simple protein precipitation (PPT) to more complex solid-phase extraction (SPE). An easy and inexpensive sample preparation method to determine multiple classes of pharmaceuticals in biological samples has been an unmet need to date. Polymeric or mixed-mode SPE sorbents can address this need by isolating acidic, neutral, and basic drugs through a combination of hydrophobic and ion-exchange interactions. However, these techniques involve multiple steps, require a firm understanding of chemical interaction, and can be expensive as well as time-consuming.

Previously, we described an extension of the work presented by Plössl; *et al.* [1] for the determination of pharmaceuticals in whole blood samples, using a modified miniextraction procedure and dispersive SPE cleanup with LC/MS/MS analysis [2]. Other researchers have also implemented a mini-QuEChERS approach with matrix cleanup by dispersive SPE [3,4,5]. The results are promising but a major disadvantage, noted by several authors, was the lack of lipid removal from the biological matrix. Coextracted lipids can present major issues during analysis including contamination of the chromatographic system and MS ion suppression. Various sorbents or interference scavengers have been evaluated. These include C18 and graphitized carbon [3]. C18 sorbent was found to have a negligible effect in removing lipids, and graphitized carbon was found to be the most effective. However, the use of graphitized carbon considerably lowered recoveries of some analytes due to nonselective removal of hydrophobic and planar compounds.

In this application note, we extend the original Enhanced Matrix Removal—Lipid (EMR—Lipid) methods for the extraction of compounds from food types [6] to include lipid removal from a biological matrix. The experiments presented in this application note used human plasma from various donors containing either NaEDTA or NaCitrate, anticoagulant agents. Protein precipitation was performed with acidified acetonitrile, followed by cleanup with EMR—Lipid and enhanced post sample treatment (polish and dry step). The experiments were performed using 25 different toxicologically monitored compounds with a broad range of hydrophobicity (Log P) and pKa (Table1).

Table 1. Test compounds.

Compound	CAS number	Log P	pKa	Therapeutic use
Alprazolam	028981-97-7	3.02	1.79, 5.08	Anxiolytic drug, sedative
Amphetamine	000051-63-8	1.80	10.01	CNS stimulant
Cocaine	000053-21-4	2.28	8.85	Local anesthetic, vasoconstrictor agent
Codeine	000076-57-3	1.34	9.19	Antitussive, opioid analgesic
Diazepam	000439-14-5	3.08	2.92	Anxiolytic agent, muscle relaxant, sedative
Heroin	005893-91-4	1.55	9.10	Opioid analgesic
Hydrocodone	000143-71-5	1.96	8.61	Antitussive, opioid analgesic
Lorazepam	000846-49-1	3.53	12.46	Anxiolytic drug, sedative
MDA 3,4-methylenedioxyamphetamine hydrochloride	013673-99-9	1.43	10.01	Psychoactive drug
MDEA 3,4-methylenedioxyl-N-ethyl-amphetamine hydrochloride	116261-63-3	2.22	10.22	Psychoactive drug
MDMA 3,4-methylenedioxymethamphetamine hydrochloride	092279-84-0	1.86	10.14	Stimulant, psychedelic drug
Meperidine hydrochloride	000050-13-5	2.46	8.16	Opioid analgesic
Methadone	001095-90-5	5.01	9.12	Antitussive, opioid analgesic
Methamphetamine hydrochloride	000051-57-0	2.24	10.21	CNS stimulant
Nitrazepam	000146-22-5	2.89	2.60, 3.28	Anticonvulsant, anxiolytic drug, sedative
Oxazepam	000604-75-1	2.92	10.61, 12.47	Anxiolytic drug, sedative
Oxycodone	000124-90-3	1.03	8.21	Antitussive, opioid analgesic
PCP Phencyclidine hydrochloride	000956-90-1	4.49	10.64	Intravenous anesthetic
Phentermine	000122-09-8	2.08	10.25	CNS stimulant
Proadifen	000062-68-0	5.61	8.96	Drug metabolism inhibitor
Strychnine	000057-24-9	0.93	9.27	Pesticide, rodenticide
Temazepam	000846-50-4	2.79	10.68	Anxiolytic drug, sedative
THC	001972-08-3	5.94	9.34	Analgesic, antiemetic, psychotropic drug
Trazodone	025332-39-2	3.13	7.09	Antidepressant, anxiolytic drug
Verapamil	000152-11-4	5.04	9.68	Ca channel blocker, vasodilator agent

Experimental

All reagents and solvents were HPLC analytical grade Honeywell (Muskegon, MI, USA). Formic acid (FA) and the toxicological mix were purchased from Agilent Technologies. The deuterated internal standards were purchased at a 100 µg/mL solution in methanol from Sigma-Aldrich (St. Louis, MO, USA). The deuterated internal standard working solution (IS-WS) mix was prepared at 1 µg/mL in acetonitrile. The internal standard mix contained amphetamine-d5, diazepam-d5, lorazepam-d4, and phencyclidine-d5. The 25 compound 1 µg/mL solution was diluted with acetonitrile to make the following intermediate working solutions (WS):

For prespiking QC samples

- L-WS: 100 ng/mL
- M-WS: 500 ng/mL
- H-WS: 1,000 ng/mL; use stock standard (SS) without dilution

For post spike solutions made at 10 times the final concentration

- Cal-1: 10 ng/mL
- Cal-2: 20 ng/mL
- Cal-3: 200 ng/mL
- Cal-4: 500 ng/mL; use M-WS
- Cal-5 and Cal-6: 1,000 ng/mL; use H-WS

Consumables and equipment

- Agilent 1290 Infinity LC with Diode Array Detector
- Agilent 6490 Triple Quadrupole LC/MS with Agilent Jet Stream and iFunnel Technology
- Agilent Technologies Formic Acid (FA) (p/n G2453-85060)
- Agilent Technologies LC/MS Toxicology Test Mixture, 1 µL/mL (p/n 5190–0470)
- Bond Elut Enhanced Matrix Removal—Lipid dSPE, 1 g (p/n 5982–0101)
- Ceramic homogenizers for 2 mL tubes (p/n 5982–9311)
- Bond Elut Polish Pouch, $MgSO_4$, 3.5 g (p/n 5982–0102)
- Plasma (Biological Specialty Corporation, Colmar, PA USA)
- Water OASIS Prime HLB 3 cc (60 mg) cartridges (186008056)
- Phenomenex Phree Phospholipid Removal Tabbed 1 mL tubes (8B-S133-TAK)
- Supelco HybridSPE Phospholipid Ultra (30 mg) 1 mL SPE tubes (55269-U)
- Sorvall ST 16 R Centrifuge (Thermo IEC, MA, USA)
- Micro centrifuge 5415D Eppendorf (Brinkman Instruments, Westbury, NY USA)
- VWR Vortex Mixer, variable speed (VWR International, West Chester, PA, USA)
- DVX 2500 Multi-Tube Vortexer (VWR International, West Chester, PA, USA)

Table 2 lists the other MS conditions relating to the analytes.

HPLC conditions

Parameter	Value
Column:	Agilent Poroshell 120 EC-C18: guard 2.1 × 5 mm (p/n 821725-911), column 2.1 × 100 mm, 2.7 µm (p/n 695775-902)
Flow rate:	0.5 mL/min
Column temperature:	60 °C
Injection volume:	2 µL
Mobile phase:	A) 5 mM ammonium formate, 0.01% formic acid (1 L water + 0.3153 g ammonium formate + 0.1 mL formic acid) B) Acetonitrile, 0.01% formic acid (1 L acetonitrile + 0.1 mL formic acid)
Gradient:	Time (min) %B 0.0 10 0.5 15 3.0 50 4.0 95 6.0 95
Gradient program for lipid evaluation:	Time (min) %B 0.0 5 3.0 30 17.0 100 30.0 100

MS conditions

Source parameters	
Ion source:	AJS ESI, Positive
Dynamic MRM	
Gas temperature:	120 °C
Gas flow:	14 L/min
Nebulizer:	40 psi
Sheath gas temperature:	400 °C
Sheath gas flow:	12
Capillary:	3,000 V
VCharging:	0
Ion funnel parameters	
POS High-Pressure RF:	90
POS Low-Pressure RF:	70

MS conditions for lipid evaluation

Source parameters	
Same as noted previously	
Precursor ion scan	
Product ion:	184
MS1 from:	100
MS1 to:	1,000
Scan time:	40
Frag mode:	Fixed
Frag:	380 V
CE:	40 V
Cell acc:	7 V

Table 2. MS parameters for the test compounds.

Compound*	Precursor ion	Product ion	CE voltage	Retention time (min)	Retention window
Codeine (A)	300.2 300.2	128.1 165.1	40 60	1.15	0.5
Oxycodone (A)	316.2 316.2	298.2 256.1	16 24	1.47	0.7
Amphetamine (A)	136.1 136.1	91.1 119.1	20 10	1.48	0.5
DL-Amphetamine-d5 (IS-A)	141.1 141.1	93.1 124.1	20 10	1.47	0.5
MDA (A)	180.1 180.1	163.1 135.1	4 16	1.54	0.5
Hydrocodone (A)	300.2 300.2	199.1 171.1	28 40	1.67	0.7
Methamphetamine (A)	150.1 150.1	119.1 91.1	8 20	1.72	0.5
MDMA (A)	194.1 194.1	163.1 135.0	8 20	1.74	0.5
Strychnine (A)	335.2 335.2	184.1 156.1	40 40	1.95	0.5
Phentermine (A)	150.1 150.1	133.0 65.1	8 48	1.96	0.5
MDEA (A)	280.1 208.1	163.1 135.1	8 20	2.05	0.5
Heroin (L)	370.2 370.2	165.1 328.2	40 20	2.60	0.5
Cocaine (L)	304.2 304.2	182.1 91.0	16 48	2.72	0.5
Meperidine (L)	248.2 248.2	220.1 174.1	20 16	2.77	0.5
Trazodone (P)	372.2 372.2	176.1 148.1	24 36	3.12	0.5
PCP (P)	244.2 244.2	159.1 91.1	8 36	3.21	0.5
PCP-d5 (IS-P)	249.2 249.2	96.1 86.1	40 10	3.20	0.5
Oxazepam (L)	287.1 287.1	241.1 269.1	20 12	3.79	0.5
Nitrazepam (L)	282.1 282.1	236.1 190.1	24 56	3.79	0.5
Verapamil (L)	455.3 455.3	165.1 303.2	28 24	3.90	0.5
Lorazepam (L)	321.0 321.0	275.0 303.0	20 12	3.88	0.5
Lorazepam-d4 (IS-L)	325 325	279.0 233.1	20 40	3.88	0.5
Methadone (L)	310.2 310.2	265.2 219.1	12 20	3.98	0.5
Alprazolam (L)	309.1 309.1	205.1 281.1	48 40	4.01	0.5
Temazepam (L)	301.1 301.1	255.1 283.1	16 8	4.21	0.5
Proadifen (D)	354.2 354.2	209.1 167.1	20 40	4.48	0.5
Diazepam (D)	285.1 285.1	193.1 257.1	32 20	4.57	0.5
Diazepam-d5 (IS-D)	290.1 290.1	198.1 154.0	40 40	4.56	0.5
THC (L)	315.2 315.2	193.1 135.1	20 20	5.67	0.5

* The letter after the name of each compound represents the corresponding deuterated internal standard that was used for quantitation.

General procedure

Prespiked plasma

1. Pipette 500 µL of plasma + QC (25 µL) + IS (25 µL) + 950 µL ACN (0.2% FA) into a 5 mL centrifuge tube.
2. Vortex, centrifuge at 5,000 rpm, 5 minutes.
3. Add 500 µL water to EMR—L 200 mg sorbent in a 5 mL centrifuge tube, vortex.
4. Decant entire extract into EMR—L tube, vortex, centrifuge at 5,000 rpm, 5 minutes.
5. Dispense extract into empty 5 mL tube, add 450 mg $MgSO_4$, vortex immediately, centrifuge at 5,000 rpm, 5 minutes.
6. Transfer supernatant to 2 mL tube containing ~125 mg $MgSO_4$, vortex immediately, centrifuge at 13,000 rpm, 3 minutes.
7. Take 200 µL of final extract + 800 µL of water into a 2 mL AS vial, vortex, analyze.

Blank plasma

1. Pipette 2.5 mL of plasma + 5 mL of ACN (0.2% FA) into a 15 mL centrifuge tube
2. Vortex, centrifuge at 5,000 rpm, 5 minutes.
3. Add 2.5 mL of water to EMR—Lipid 1 g (15 mL centrifuge tube), vortex.
4. Decant the entire extract, vortex, centrifuge at 5,000 rpm, 5 minutes.
5. Dispense extract into empty 15 mL tube, add contents of Polish pouch, shake and vortex immediately, centrifuge at 5,000 rpm, 5 minutes.
6. Transfer supernatant to empty 15 mL tubes, add 1.2 g $MgSO_4$, vortex, centrifuge.
7. Transfer blank matrix to a clean vial for postspiked matrix matched calibrations, see experimental section.
8. Calibration curve

Calibration level	Matrix blank extract µL	Calibrant solution	Calibrant solution µL	Internal standard working solution (IS-WS) µL
1	450	Cal-1	25	25
2	450	Cal-2	25	25
3	450	Cal-3	25	25
4	450	Cal-4	25	25
5	450	Cal-5	25	25
6	425	Cal-6	50	25

9. To produce the individual calibration levels, combine the mentioned components into a test tube, vortex.
10. Take 200 µL of postspiked matrix at each calibration level + 800 µL of water into a 2 mL AS vial, vortex, analyze.

Results and Discussion

Matrix effects

Protein precipitation is a commonly used sample preparation technique with biological samples. Although it does remove proteins from the extract, lipid coextraction with target analytes can cause chromatographic anomalies and MS ion suppression. Agilent Bond Elut EMR—Lipid selectively removes lipids through size exclusion and hydrophobic interactions without affecting analyte recovery. Phospholipids were monitored by LC/MS/MS using precursor ion scan for product ion *m/z* 184 and MS1 from 100 to 1,000. The chromatography run was extended out to 30 minutes to elute matrix coextractives remaining on the column and possibly in the ion source. These coextractives would not necessarily be apparent in a short fast gradient used for analyte analysis, but can elute in subsequent injections, causing analytical variability. Figure 1 shows the overlay chromatogram for a plasma sample with protein precipitation, and after protein precipitation with EMR—Lipid dSPE cleanup using two different human plasma samples and different anticoagulants.

To compare the lipid removal efficiency of EMR—Lipid to other commercially available phospholipid removing sorbents, an aliquot of 500 µL of plasma was mixed with 1 mL of ACN (0.2% FA) to precipitate the proteins, and then passed through the phospholipid cartridges by vacuum. Figure 2 demonstrates the remarkably efficient lipid cleanup of EMR—Lipid when compared to common phospholipid-removing sorbents: Waters Oasis Prime, Phenomenex Phree, and Supleco HybridSPE. Figure 3 is the representative chromatographic overlay of the dynamic MRM for a 10 ng/mL spike plasma sample after protein precipitation and EMR—Lipid dispersive SPE cleanup.

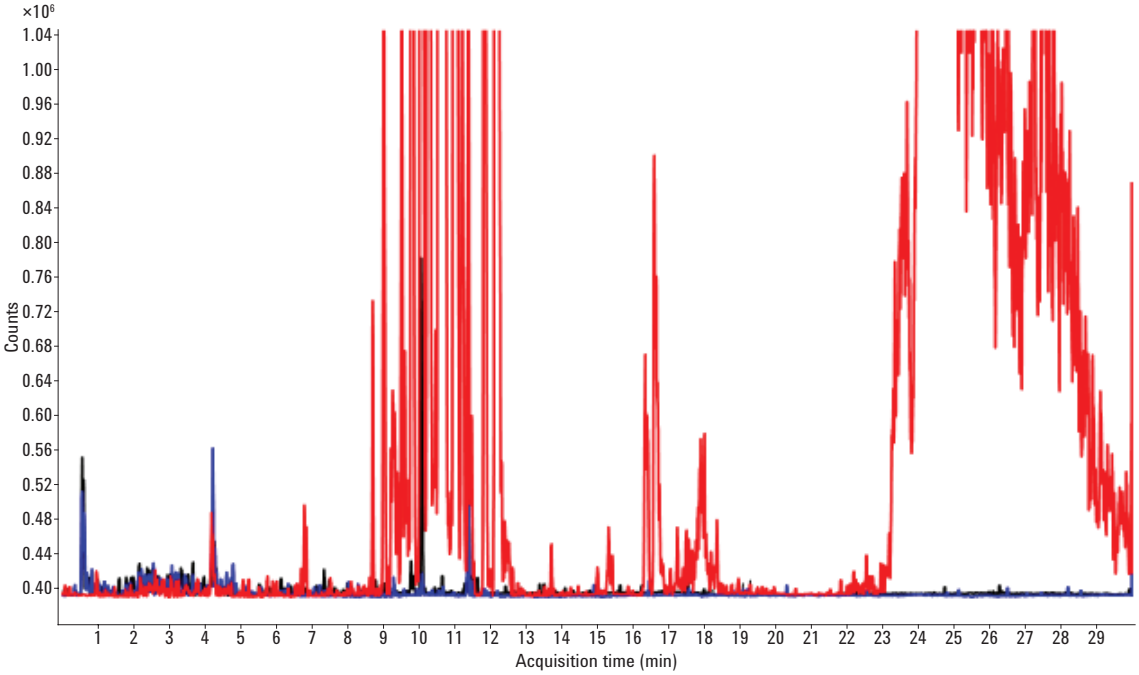

Figure 1. Overlay of plasma sample after protein precipitation (red) and plasma sample after protein precipitation and EMR—Lipid dSPE cleanup on two separate plasma samples (blue and black). LC/MS/MS product ion *m/z* 184.

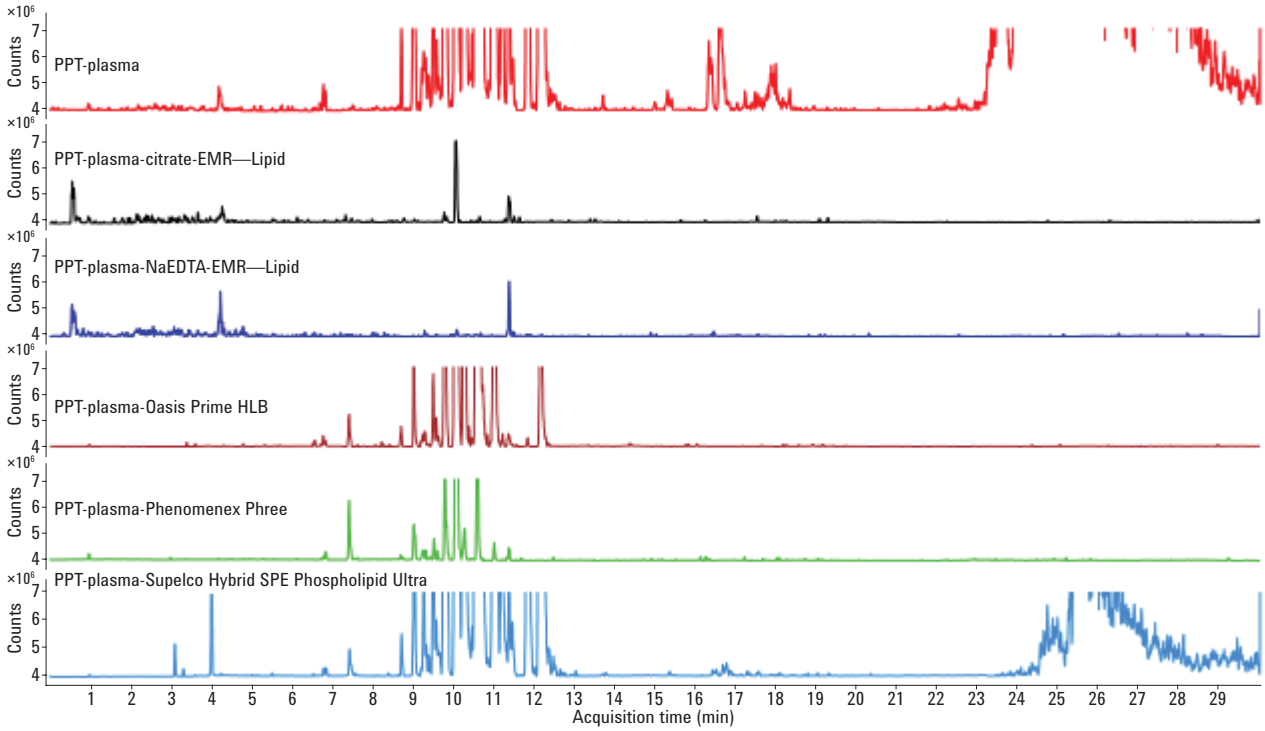

Figure 2. LC/MS/MS product ion *m/z* 184 for plasma sample after protein precipitation and various lipid removal sorbents.

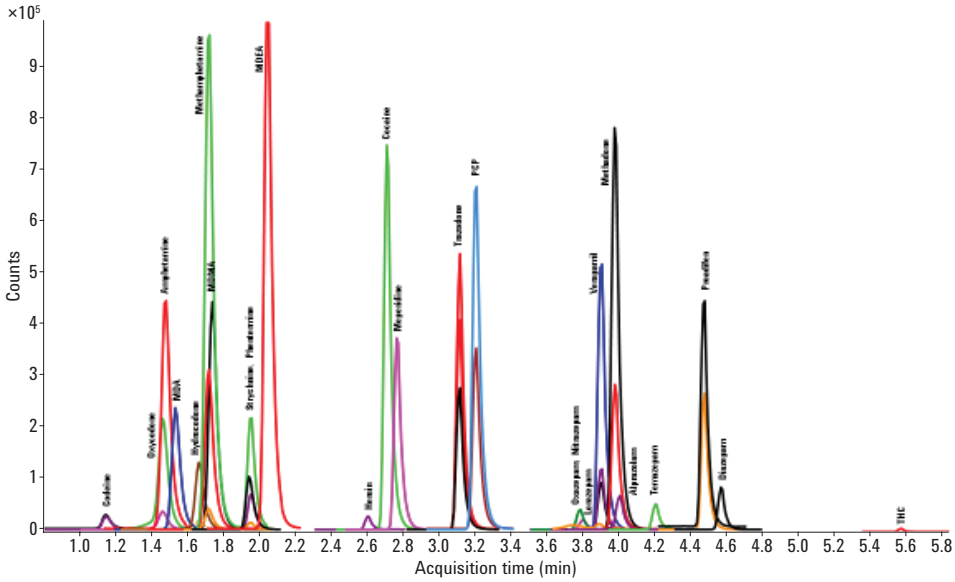

Figure 3. LC/MS/MS MRM chromatogram of 10 ng/mL spike plasma sample after protein precipitation and EMR—L dSPE cleanup.

Linearity and limit of quantification (LOQ)

The linear calibration range evaluated for all the toxicological compounds was 0.5 to 100 ng/mL. Matrix blank plasma extracts were prepared for the calibration curves. The matrix blank was spiked at the appropriate calibration levels (see experimental and general procedure) for final concentrations of 0.5, 1, 10, 25, 50, and 100 ng/mL. The internal standard mix was spiked at a 50 ng/mL concentration. The calibration standards were run in replicates of six (n = 6), and generated by plotting the relative responses of analytes (peak area of analyte/peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). Table 3 shows the correlation coefficient (R^2) and LOQ based on method performance for the individual toxicological compounds used in the study. All compounds are below the reference range of 15–75 ng/mL.

Table 3. Linear correlation coefficient and LOQ for toxicological compounds in this study.

Compound	R^2	LOQ (ng/mL)
Codeine	0.9989	1.0
Oxycodone	0.9988	0.5
Amphetamine	0.9991	0.5
MDA	0.9916	0.5
Hydrocodone	0.9984	0.5
Methamphetamine	0.9983	0.5
MDMA	0.9989	0.5
Strychnine	0.9993	0.5
Phentermine	0.9990	0.5
MDEA	0.9857	0.5
Heroine	0.9967	0.5
Cocaine	0.9766	0.5
Meperidine	0.9952	0.5
Trazodone	0.9983	0.5
PCP	0.9991	0.5
Nitrazepam	0.9919	0.5
Oxazepam	0.9966	1.0
Lorazepam	0.9964	5.0
Verapamil	0.9915	0.5
Methadone	0.9736	0.5
Alprazolam	0.9939	0.5
Temazepam	0.9961	0.5
Proadifen	0.9988	0.5
Diazepam	0.9996	0.5
THC	0.9929	5.0

Accuracy and reproducibility

The accuracy and reproducibility was determined by spiking standards into the plasma samples at 5, 25, and 50 ng/mL, in replicates of five (n = 5). These QC plasma samples were then subjected to protein precipitation and EMR—Lipid dispersive SPE cleanup. Accuracy was determined against the matrix matched calibration curve. Figures 4 and 5 show the recovery and relative standard deviation.

The results show excellent recoveries for all compounds with average recoveries at 5 ng/mL greater than 95% and RSD <6%. Heroin was the only compound with relatively lower recoveries 65% on average, but RSDs below 8%.

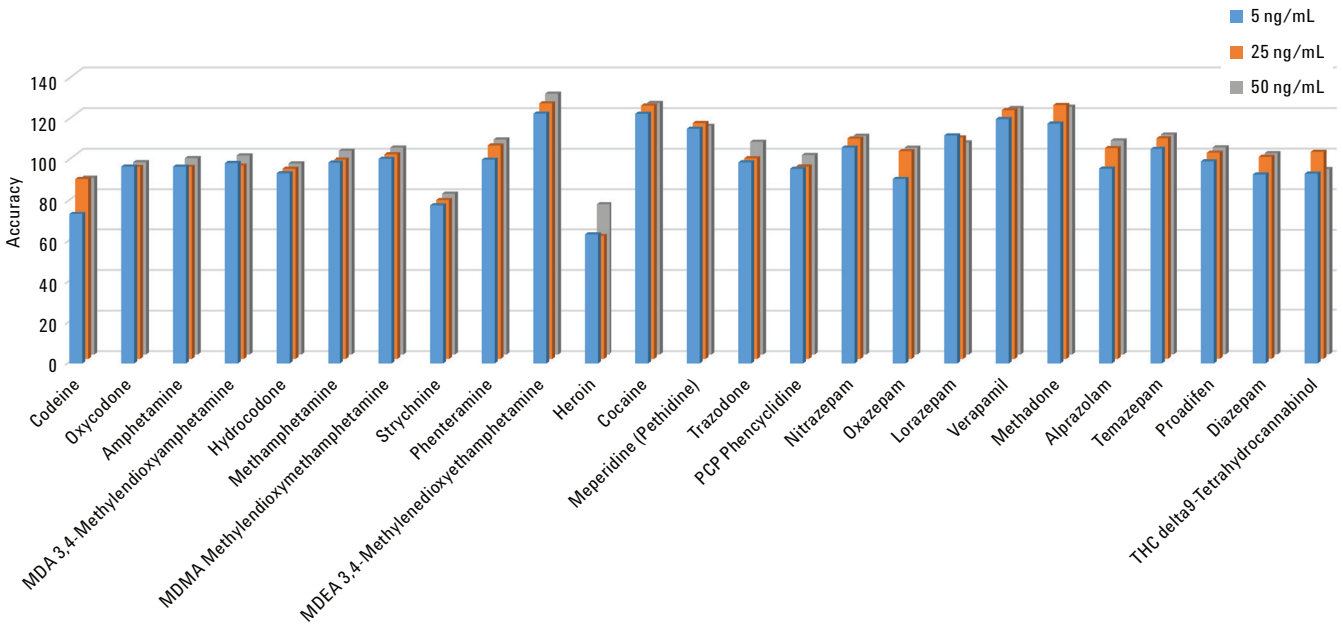

Figure 4. Accuracy for 25 extracted toxicological compounds from human plasma.

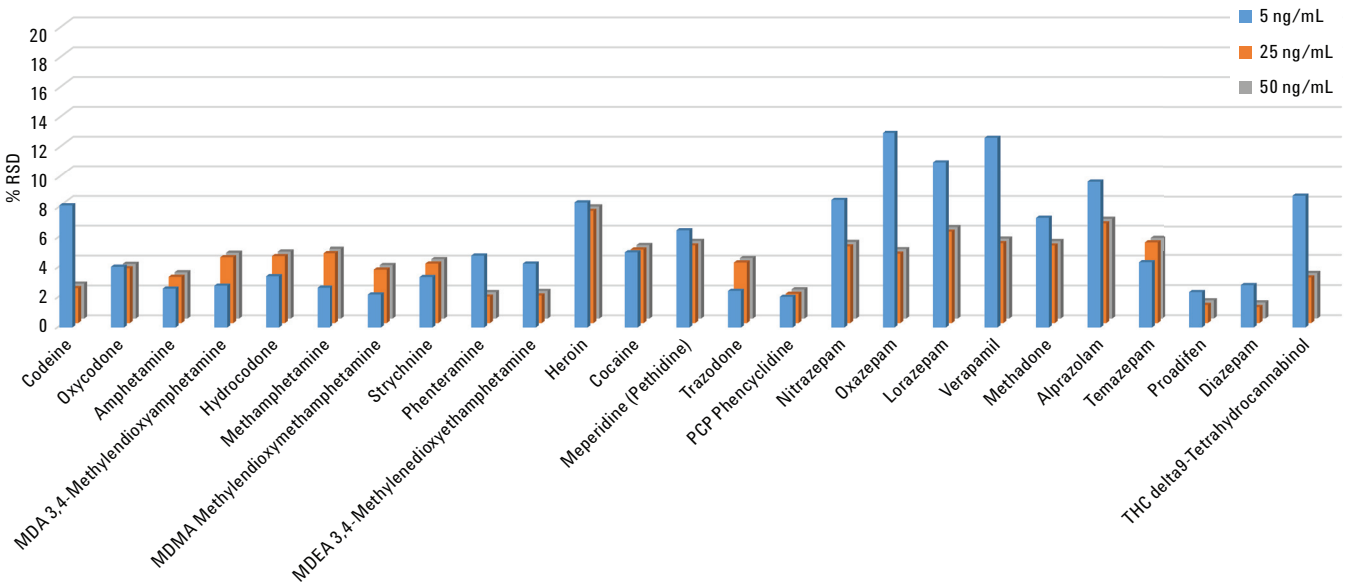

Figure 5. Relative standard deviations (RSDs) for 25 toxicological compounds in human plasma.

Conclusion

A simple and inexpensive sample cleanup method using protein precipitation and EMR—Lipid dispersive SPE cleanup has been developed for plasma LC/MS/MS for a range of toxicological compounds. The advantages of this new method are:

- A cleaner extract with significant overall lipid removal compared to standard PPT and commercially available lipid removal products.
- Excellent recoveries and single digit % RSDs.
- Substantially cleaner extracts without the need for extra instrumentation or glassware, offering an easy and user-friendly sample preparation.

This approach for the extraction of toxicological compounds from plasma is easily implemented into laboratories without extensive expertise in sample preparation techniques since it only involves spiking, vortexing, and centrifugation.

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