

## Application Note

# Providing a Universal, One-step Alternative to Liquid-Liquid Extraction in Bioanalysis

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## Abstract

This application note demonstrates the effectiveness of Ostro plate technique and its broad applicability in bioanalytical assays by comparing the Ostro sample preparation plate and a generic LLE method for the extraction of a diverse group of pharmaceutical drugs.

### Benefits

- Simple, one-step sample preparation method
- Universal protocol requiring no method development
- Removal of >95% of residual phospholipids relative to LLE with MTBE
- Eliminates extract transfer and evaporation steps compared to traditional LLE
- Reduces sample variability and eliminates sources of suppression

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## Introduction

Bioanalytical methods are under constant pressure from regulatory agencies to meet requirements for matrix effects, accuracy and precision, more challenging lower limits of quantitation (LLOQ) and incurred sample reanalysis (ISR). Sample preparation has become an increasingly important aspect of meeting these challenges. The introduction of more sensitive mass spectrometers has further increased the burden on sample preparation and on the generation of the cleanest extracts possible. Liquid-liquid extraction (LLE) is a common sample preparation choice in regulated bioanalysis. LLE can generate high analyte recoveries, clean extracts, and is perceived as low cost. Depending on the compounds analyzed, however, LLE methods must often be optimized. Extraction solvents may need to be acidified, basified or low percentages of more polar solvents may be required to simultaneously achieve high recoveries for metabolites and related compounds, as well as the primary analyte. In many cases, the choice of LLE solvent may lead to extracts particularly saturated with phospholipids (PLs). As well as contributing to matrix effects, residual PLs can build-up on the analytical column and the LC system. PL build-up may cause analyte signal variability, suppression of MS response, and potentially lead to instrument down-time. Similar to LLE, phospholipid removal (PLR) plates also provide high analyte recoveries and clean extracts. In addition to these benefits, the Ostro 96-well sample preparation plate provides a simple, one-step method which achieves high recoveries for diverse

analyte types without optimization. In addition, the need to transfer, evaporate, and reconstitute the extract is eliminated, thus, significantly decreasing the sample preparation time. To demonstrate the effectiveness of this technique and its broad applicability in bioanalytical assays, the Ostro sample preparation plate and a generic LLE method were compared for the extraction of a diverse group of pharmaceutical drugs. With no method development, analyte recovery was high, >75%, for polar and nonpolar, acidic, and basic analytes alike, and 95% of PLs were removed relative to LLE. Ostro extraction of 96 samples was achieved in half the time relative to 96-well LLE and in less than 1/10th the time if LLE was performed in individual tubes.

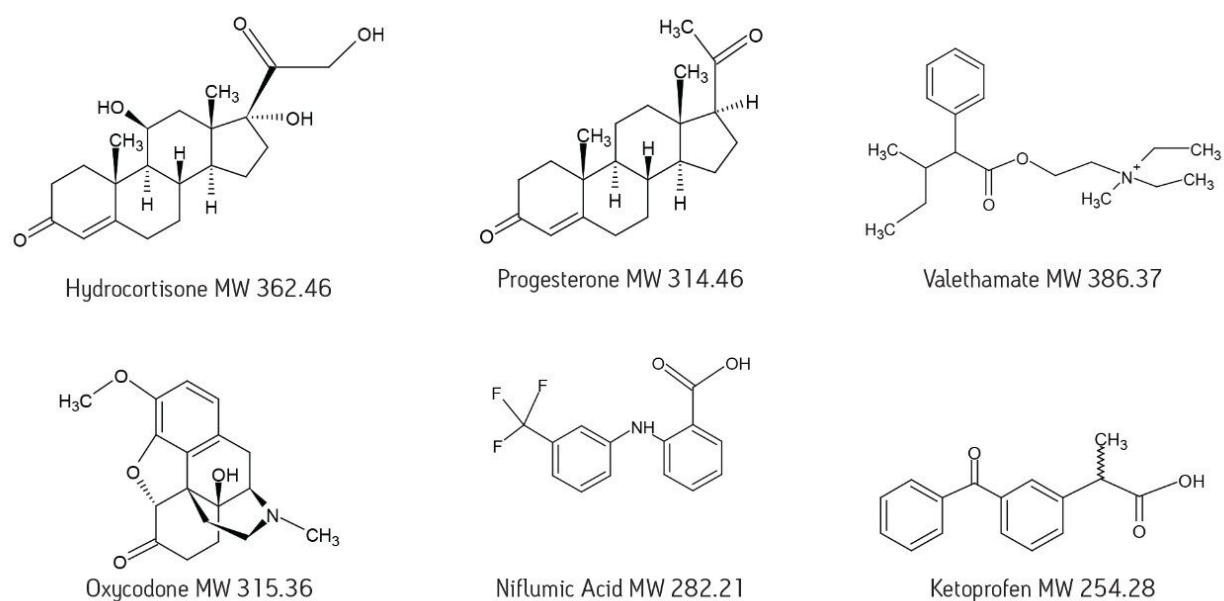


Figure 1. Representative structure and molecular weights of the selected drug compounds used in this study.

## Experimental

### ACQUITY UPLC Conditions

Column: ACQUITY UPLC BEH C<sub>18</sub>, 2.1 x 50 mm, 1.7  $\mu$ m

Mobile Phase A: 0.1% NH<sub>4</sub>OH in H<sub>2</sub>O

Mobile Phase B:	Acetonitrile
Flow Rate:	0.6 mL/min
Injection Volume:	30.0 µL
Injection Mode:	Partial Loop
Column Temperature:	35 °C
Sample Temperature:	15 °C
Strong Needle Wash:	60:40 ACN:IPA + 0.2% conc. HCOOH (600 µL)
Weak Needle Wash:	80/20 H <sub>2</sub> O/MeOH (200 µL)

## Gradient

Time (min)	Profile		Curve
	%A	%B	
0.0	95	5	6
2.0	2	98	6
3.0	2	98	6
3.1	95	5	6
3.5	95	5	6

## Waters Xevo TQ-S Conditions, ESI+

Capillary Voltage:	3.0 kV
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Desolvation Temp:	550 °C
Cone Gas Flow:	150 L/Hr
Desolvation Gas Flow:	1000 L/Hr
Collision Cell Pressure:	$2.6 \times 10^{-3}$ mbar
MRM transition monitored, ESI+:	See Table 1

Analyte	Precursor Mass	Daughter Mass	Cone Voltage (V)	Collision Energy (eV)
Oxycodone	316.3	256.1	30	28
Valethamate	306.2	163.0	34	24
Niflumic Acid	283.3	265.1	34	20
Ketoprofen	255.3	209.2	28	14
Progesterone	315.1	96.6	34	20
Hydrocortisone	363.1	327.1	36	14

*Table 1. MRM transitions, collision energies, and cone voltages for a diverse group of drug compounds.*

## Sample Preparation Protocol

Extraction using the Ostro plate was performed using 200 µL of pre-spiked or blank rabbit plasma which was extracted with 600 µL of 1% HCOOH in acetonitrile (Figure 2). LLE was performed using 200 µL of pre-spiked or blank rabbit plasma and extracted with 1 mL of 100% MTBE in a centrifuge tube. The tube was vortexed for 1 minute, centrifuged at 3500 rpm for 5 minutes, and supernatant transferred to a 2 mL 96-well plate. To facilitate direct comparison, all extracts were post-spiked and dried under nitrogen gas in order to determine recovery. The samples were reconstituted in a constant volume of 200 µL 50/50 MeOH/H<sub>2</sub>O prior to injection onto the LC-MS/MS system so that the Ostro plate and LLE sample results could be compared to one another. Eluates from the Ostro plate were usually direct injected.

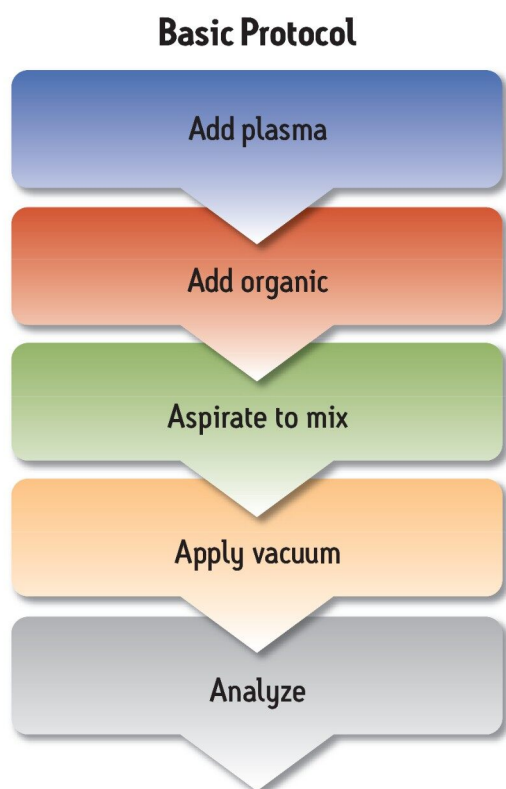
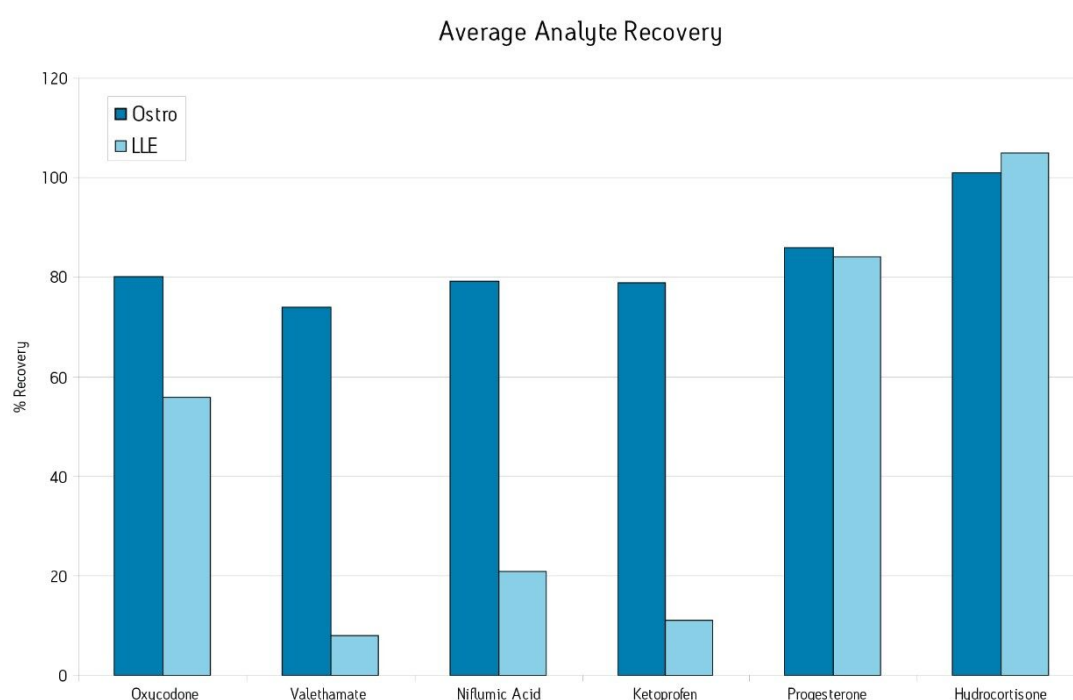


Figure 2. Ostro plate basic plate protocol.

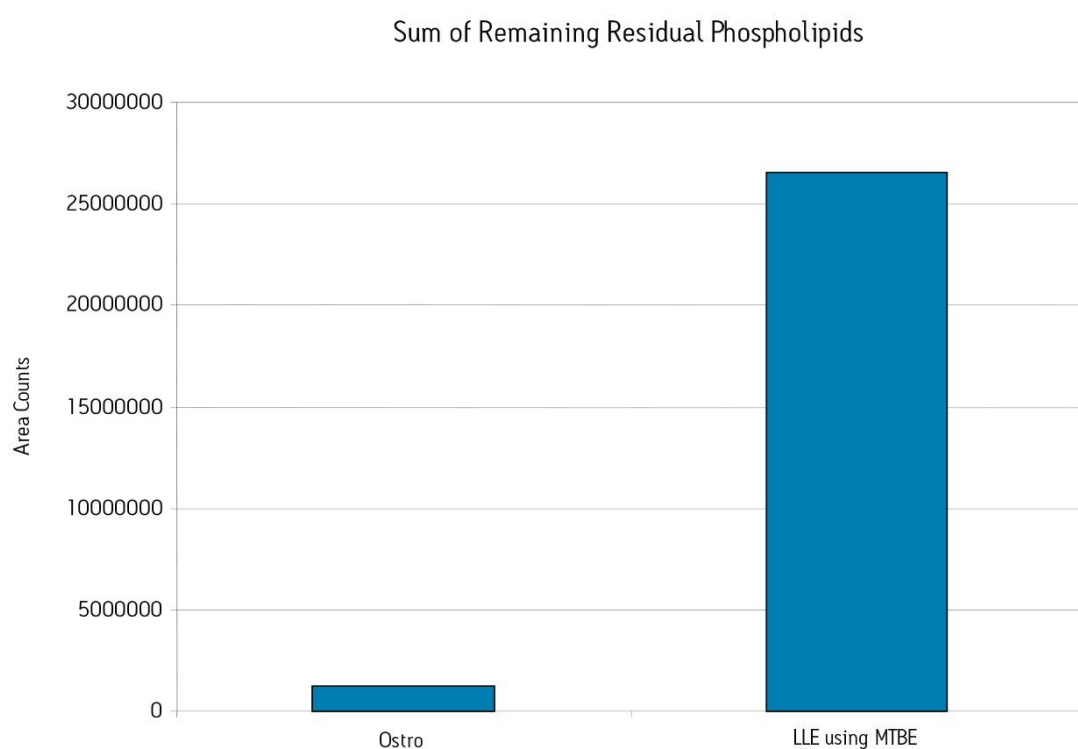
## Results and Discussion

Recovery was calculated for both the Ostro plate and LLE and the averages (n=8) were compared for the group of drug compounds (Figure 3). The average recovery for the Ostro plate across all analytes was 83%. The average for LLE across all analytes was 47%. The levels of PLs remaining after each sample preparation technique were also compared. This was accomplished by quantifying five common PLs from the Ostro and LLE extracts. Area counts were summed, and the resultant data indicated that >95% of PLs were removed relative to LLE (Figure 4). To visually demonstrate the remaining levels of residual phospholipids, the MRM transition 184>184 is shown for both traditional LLE and extraction in-well using the Ostro plate (Figure 5). For the cleanest samples, Ostro sample preparation plates can be combined with LLE. LLE was performed as previously discussed and the supernatant passed through Ostro plates. The resulting eluate was then dried down and reconstituted following the original method. This combined sample prep method resulted in the removal of >99.9% of residual PLs (Figure 6) relative to LLE alone. To demonstrate the effect these residual

PLs can have on signal variability, the peak area for a representative compound was monitored during overnight analytical runs of Ostro and LLE extracts. The resulting %RSD values were 5% for Ostro samples and 25% for LLE samples. This signal variability observed in LLE samples may be detrimental when performing incurred sample reanalysis (ISR) to fully validate the drug method. In bioanalysis, high throughput is also important. A time table was created to compare sample preparation time for 96 samples extracted using an Ostro plate, LLE in a 96-well plate, and traditional LLE in centrifuge tubes (Figure 7). The total time required to prepare 96 plasma samples was 11 minutes using the Ostro plate, 21 minutes for LLE in 96-well format, and 164 minutes for LLE samples in individual centrifuge tubes.



*Figure 3. Average analyte recoveries for a mix of drug compounds comparing the Ostro sample preparation plate to LLE with 100% MTBE.*



*Figure 4. Comparison of the sum of phospholipid levels between samples extracted in-well using the Ostro 96-well plate and samples extracted using the traditional LLE method in tubes  $n=8$  for each technique. The 5 phospholipids summed have precursor masses of 496, 524, 704, 758, and 806.*



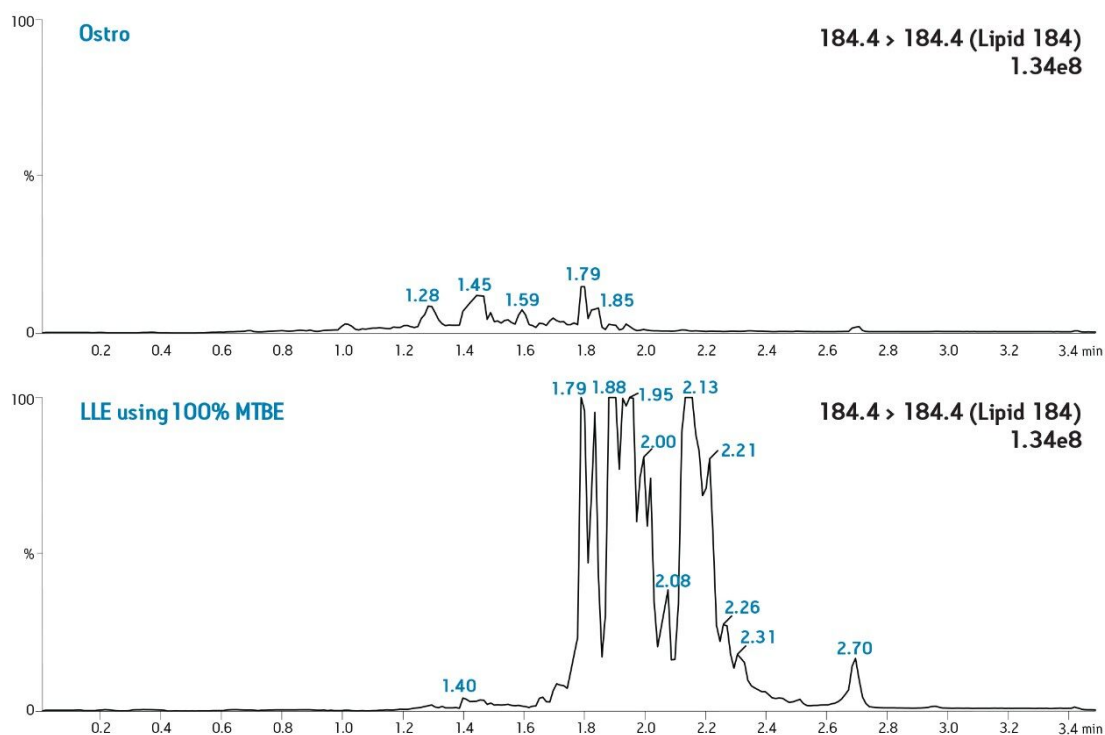


Figure 5. MRM transition 184>184 was monitored to visually demonstrate total remaining residual PLs from the Ostro plate and LLE using 100% MTBE.

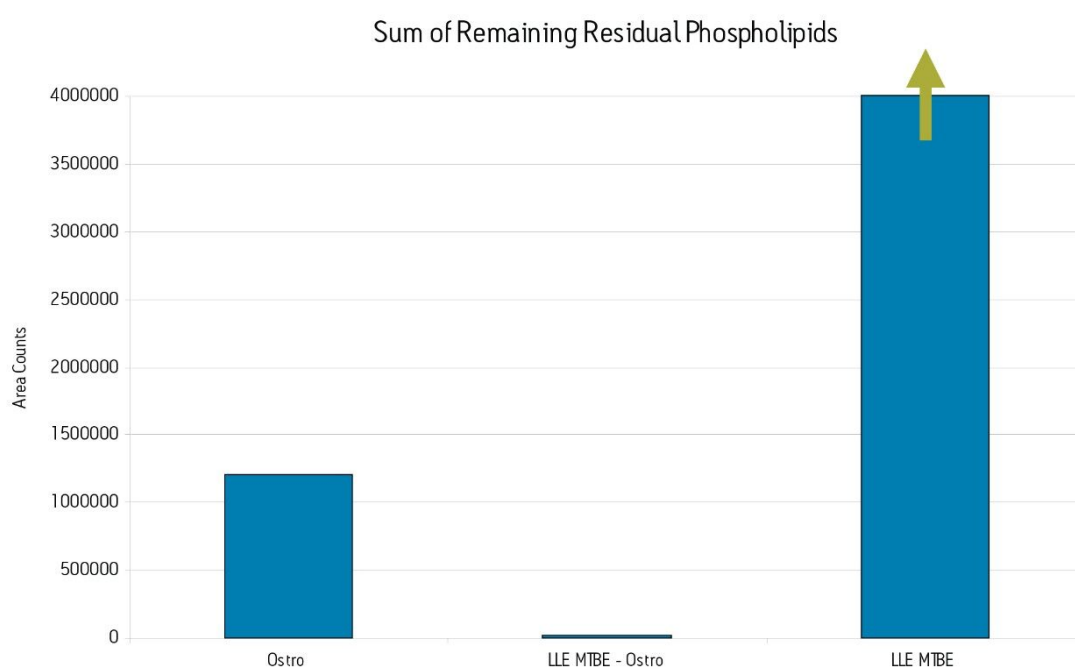


Figure 6. Comparison of the sum of phospholipid levels between samples extracted in-well using the Ostro 96-well plate and samples extracted using the traditional LLE method in tubes  $n=8$  for each technique. The 5 phospholipids summed have precursor masses of 496, 524, 704, 758, and 806.



Figure 7. A comparison of the time required to prepare 96 plasma-based samples using an Ostro 96-well plate, LLE in 96-well format, and LLE in centrifuge tubes. Four LLE tubes were vortexed simultaneously. Pipetting for the 96-well plates was performed using a multi-channel pipette.

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## Conclusion

- Ostro 96-well sample preparation plates,
  - are a simple, one-step sample prep alternative to LLE in bioanalysis.
  - utilize a generic method that requires no method development for analytes with diverse chemical properties.
  - allow for direct injection of eluates, which eliminates the evaporation step required for LLE extracts.
  - remove >95% of residual phospholipids when compared to traditional LLE.
  - provide for a significant reduction in sample prep time relative to LLE in 96-well format or LLE in individual tubes.
- A combination of the Ostro plate and LLE removes >99.9% of residual phospholipids.

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