SPOTLIGHT feature

Chromatography

# Supported Liquid Extraction versus Liquid-Liquid Extraction: Extracting Acidic, Basic and Neutral Analytes from Plasma

James Edwards, Porvair Sciences, james.edwards@porvairsciences.com

Sample preparation is a key process when running any samples on a chromatography instrument. No matter how good the instrument is, to get the best sensitivity and reproducibility sample clean-up needs to be performed. A traditional method has been to use liquid-liquid extraction (LLE) which allows transfer of analyte from an aqueous solution to an organic solution. However, LLE can have issues around the time, irreproducibility and emulsions forming.

This application note discusses the advantages of using a supported liquid extraction (SLE) over an LLE method for extracting a range of analytes (acidic, basic and neutral) from pig plasma. The Microlute™ SLE plate offers an alternative method to LLE which follows the same principles of LLE methods. It improves recovery, reproducibility and speeds up sample preparation to allow a greater throughput of samples tested

## Introduction

Liquid-liquid extraction is one of the oldest and most established sample preparation method. LLE was first developed by the petroleum industry back in 1909 for the removal of aromatic hydrocarbons from kerosene [1].

The principles underlying LLE are well known and the number of publications simplifies finding a method. LLE uses two different solvent phases which are immiscible with each other - typically an aqueous solution and a water immiscible solvent (e.g. dichloromethane, hexane, ethyl acetate) Shaking is then used to help drive the compound of interest from one phase to the other, usually from aqueous to an organic solution. The efficiency at which this occurs is called the partition coefficient, $K_p$ which is calculated using Equation 1.

$$K_p = \frac{[A]_2}{[A]_1}$$

*Equation 1. Equation to calculate the partition coefficient, $K_p$, where $[A]_1$ is the concentration of analyte A in phase 1 and $[A]_2$ is the concentration of analyte A in phase 2.*

In chromatography the partition coefficient is known as 'Log P of a molecule'. This applies the logarithm of Equation 1 when phase 1 is deionised water and phase 2 is n-octanol. This can provide information on how lipophilic/hydrophobic a molecule is, which is a useful measure for chromatography method development. Compounds with a low Log P are more hydrophilic which means they are more difficult to extract from aqueous solutions.

Due to the basic principles of solvent separation and easy access to common solvents, glassware, and equipment, LLE has become a popular extraction method in chromatography laboratories. However, this method has significant disadvantages. To obtain efficient partitioning between the phases, shaking is a crucial step. The action of shaking causes the surface area contact between the two solutions to become much higher and allow better transfer of analyte from one phase to the other. Insufficient mixing of the two solutions results in an inefficient LLE method. In contrast, too vigorous agitation can result in the formation of an emulsion - the formation of droplets of one solvent in the other which occurs when compounds are present which act as surfactants. The surfactant allows the two phases to interact with each other which causes an intermediate phase on the surface boundary (*Figure 1*).

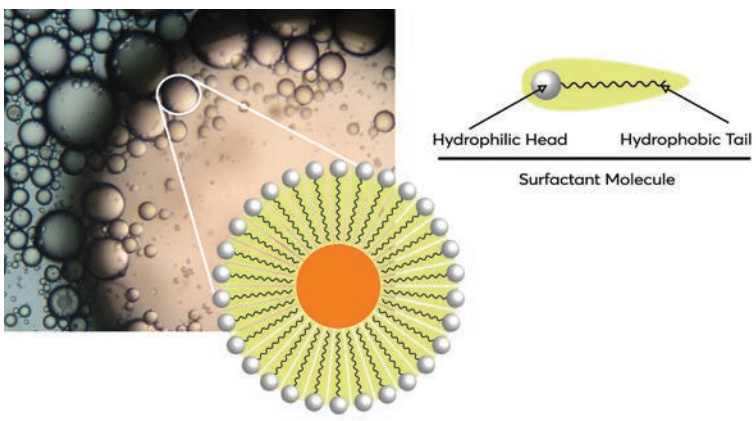



*Figure 1. Diagram of a micelle which has formed part of an emulsion. The formation of them is due to the amphipathic nature of surfactants present which have both hydrophilic heads and hydrophobic tails.*

LLE is also considered low throughput as samples are prepared serially rather than in parallel. Each step of the LLE process requires repeated agitation and transfer of solvent thereby reducing the number of samples that can be processed at any one time. Both of these steps are also influenced by different lab users which can lead to variable results.

## The Evolved Method

The alternative sample preparation based on the same principles of LLE is SLE. SLE uses diatomaceous earth as the support for the separation process to occur on. It is a naturally occurring, chemically inert porous material which has a high surface area. These properties allow water to easily load via capillary action onto the diatomaceous earth and adsorb to the surface of the diatom structures which make up the diatomaceous earth (*Figure 2*).

The SLE process mimics LLE theory with two liquid phases interacting with each other. Phase one forms when the aqueous solution (water, plasma, serum etc.) is loaded and allowed to flow on via capillary action over several minutes. This allows the water to adsorb and create a phase on the diatomaceous earth's surface which creates a very high surface area. The second phase is a water-immiscible organic solvent passed through the support bed under gravity. As the solvent flows past the adsorbed water, a partition forms with a very efficient phase boundary which acts like the shaking step in LLE. It allows sample clean-up by leaving unwanted compounds dissolved in the adsorbed water. These include compounds such as phospholipids or polar contaminants.

*Figure 2. Diatomaceous earth (DE) is composed of naturally occurring silica-based mineral made from fossilised diatoms, a class of hard-shelled algae found in seas and oceans. Its small pore size and high surface area makes it the ideal material for absorption of aqueous solutions.*

SLE is generally considered a more reproducible method compared to LLE, from a sample-to-sample, experiment-to-experiment and analyst-to-analyst basis. It eliminates all the variable steps (shaking, manual handling and throughput) associated with LLE. Efficient interactions via solvent flow under gravity remove the need for shaking and manual extraction of the solvent layer. SLE can also be easily automated by combining 96 well plates and robotic samplers, allows a vast number of samples to be processed in just one day allowing for higher throughput of samples. In this application note we will demonstrate the advantages of SLE versus LLE for both time and performance.

## Instrument Methods

**Basic Analytes LC Conditions:**

*Table 1. LC system conditions for chromatographic separation of basic analytes.*

| | |
|---|---|
| LC system | Agilent LC-MS, consisting of a 1260 LC and Single Quadrupole Mass Spectrometer.<br>Computer running chromatographic software (OpenLabs) |
| Column | Raptor Biphenyl 30 x 2.1mm, 1.8µm |
| Column temp. | 45°C |
| Injection volume | 2.00 µL |
| Flow rate | 600 µL/min |
| Mobile phase A | 0.1% Formic acid in water |
| Mobile phase B | 0.1% Formic acid in methanol |
| Solvent Composition | |

| Time (min) | A% | B% |
|---|---|---|
| 0.00 | 95.0 | 5.0 |
| 4.30 | 57.5 | 42.5 |
| 7.00 | 57.5 | 42.5 |
| 7.01 | 95.0 | 5.0 |
| 10.0 | 95.0 | 5.0 |

**Acidic Analytes LC Conditions:**

*Table 2. LC system conditions for chromatographic separation of acidic analytes.*

| | |
|---|---|
| LC system | Agilent LC-MS, consisting of a 1260 LC and Single Quadrupole Mass Spectrometer.<br>Computer running chromatographic software (OpenLabs) |
| Column | Raptor Biphenyl 30 x 2.1mm, 1.8µm |
| Column temp. | 30°C |
| Injection volume | 2.00 µL |
| Flow rate | 400 µL/min |
| Mobile phase A | 0.1% Formic acid in water |
| Mobile phase B | 0.1% Formic acid in methanol |
| Solvent Composition | |

| Time (min) | A% | B% |
|---|---|---|
| 0.00 | 45.0 | 55.0 |

**Neutral Analytes LC Conditions:**

*Table 3. LC system conditions for chromatographic separation of neutral analytes.*

| | |
|---|---|
| LC system | Agilent LC-MS, consisting of a 1260 LC and Single Quadrupole Mass Spectrometer.<br>Computer running chromatographic software (OpenLabs) |
| Column | Raptor Biphenyl 30 x 2.1mm, 1.8µm |
| Column temp. | 30°C |
| Injection volume | 2.00 µL |
| Flow rate | 400 µL/min |
| Mobile phase A | 0.1% Formic acid in water |
| Mobile phase B | 0.1% Formic acid in methanol |
| Solvent Composition | |

| Time (min) | A% | B% |
|---|---|---|
| 0.00 | 70.0 | 30.0 |
| 5.10 | 25.0 | 75.0 |
| 6.00 | 25.0 | 75.0 |
| 6.01 | 70.0 | 30.0 |
| 12.00 | 70.0 | 30.0 |

**Mass spectrometry parameters for all methods:**

*Table 4. Mass spectrometer conditions for all classes of analytes.*

| Parameter | Value |
|---|---|
| Gas Temperature | 350 °C |
| Gas Flow | 13 L/min |
| Nebuliser | 30 psi |
| Capillary Voltage | 4000 V |
| Fragmentor Voltage | 100 V |
| Scan Type | SIM |
| Ion Mode | ESI |

*Table 5. Properties for the compounds analysed - [a] Value from Drugbank [b] Predicted value from Pubchem *Compound has multiple ionisable groups present.*

| Compound | Compound Class | Formula | Molecular Mass | Log P | pKa[b] | Extraction Solvent |
|---|---|---|---|---|---|---|
| Caffeine | Neutral | $C_8H_{10}N_4O_2$ | 194.2 | -0.07[a] | 14.00 | Ethyl acetate |
| Procainamide | Base | $C_{13}H_{21}N_3O$ | 235.3 | 0.88[a] | 9.32 | 3:1 Hexane:Ethyl acetate |
| Acetaminophen | Neutral | $C_8H_9NO_2$ | 151.2 | 0.91[a] | -4.40 | Ethyl acetate |
| Hydrocortisone | Neutral | $C_{21}H_{30}O_5$ | 362.5 | 1.61[a] | 12.59, -2.80* | Ethyl acetate |
| Prednisolone | Neutral | $C_{21}H_{28}O_5$ | 360.4 | 1.62[a] | 12.59, -2.90* | Ethyl acetate |
| Pindolol | Base | $C_{14}H_{20}N_2O_2$ | 248.3 | 1.75[a] | 9.25 | Ethyl acetate |
| Dexamethasone | Neutral | $C_{22}H_{29}FO_5$ | 392.5 | 1.83[a] | 12.42, -3.30* | Ethyl acetate |
| Corticosterone | Neutral | $C_{21}H_{30}O_4$ | 346.5 | 2.02[b] | 13.86, -0.26* | Ethyl acetate |
| 4-Propylbenzoic acid | Acid | $C_{10}H_{12}O_2$ | 164.2 | 2.34[b] | 4.40 | 95:5 Dichloromethane/ IPA |
| Naproxen | Acid | $C_{14}H_{14}O_3$ | 230.3 | 3.04[b] | 4.10 | 95:5 Dichloromethane/ IPA |
| 4-Pentylbenzoic acid | Acid | $C_{12}H_{16}O_2$ | 192.3 | 3.12[b] | 4.40 | 95:5 Dichloromethane/ IPA |
| Ketoprofen | Acid | $C_{16}H_{14}O_3$ | 254.3 | 3.12[a] | 4.45 | 95:5 Dichloromethane/ IPA |
| Propranolol | Base | $C_{16}H_{21}NO_2$ | 259.3 | 3.48[a] | 9.42 | 3:1 Hexane:Ethyl acetate |
| Nortriptyline | Base | $C_{19}H_{21}N$ | 263.4 | 3.90[a] | 9.70 | 3:1 Hexane:Ethyl acetate |
| Ibuprofen | Acid | $C_{13}H_{18}O_2$ | 206.3 | 3.97[a] | 5.30 | 95:5 Dichloromethane/ IPA |
| Niflumic acid | Acid | $C_{13}H_9F_3N_2O_2$ | 282.2 | 4.43[a] | 1.90, 5.50* | 95:5 Dichloromethane/ IPA |
| Diclofenac | Acid | $C_{14}H_{11}Cl_2NO_2$ | 296.1 | 4.51[a] | 4.15 | 95:5 Dichloromethane/ IPA |
| Protriptyline | Base | $C_{19}H_{21}N$ | 263.4 | 4.70[a] | 10.50 | 3:1 Hexane:Ethyl acetate |
| Imipramine | Base | $C_{19}H_{24}N_2$ | 280.4 | 4.80[a] | 9.40 | 3:1 Hexane:Ethyl acetate |
| Desipramine | Base | $C_{18}H_{22}N_2$ | 266.4 | 4.90[a] | 10.40 | 3:1 Hexane:Ethyl acetate |
| Amitriptyline | Base | $C_{20}H_{23}N$ | 277.4 | 4.92[a] | 9.40 | 3:1 Hexane:Ethyl acetate |

## Sample Preparation Methods

**Plasma preparation**

Three separate quantities of pig plasma (Sigma-Aldrich) were spiked to a concentration of 1 µg/mL with basic, acidic and neutral compounds (*Table 5*) and allowed to equilibrate for 30 minutes before being used in both the SLE and LLE procedures.

**SLE**

Different pre-treatment solutions were used to dilute the plasma after each plasma sample was loaded into the well. This changes the pH of the solution to allow for optimal extraction with organic solvent on elution whilst also diluting samples to allow better flow onto the plate. For the pre-treatment solutions, basic compounds were diluted in 5% ammonia in water, acidic compounds in 2% formic acid in water and neutral compounds used deionised water. Neutral compounds are unaffected by pH so to ensure optimum recoveries, water was used to dilute the sample for better flow onto the Microlute™ SLE plate. 200 µL of the diluted plasma (100 µL of pig plasma + 100 µL of pre-treatment solution) was loaded onto the Microlute™ SLE 200 mg plate (Cat no: PSLE200P-001) using 3 PSI of positive pressure for 5 seconds (*Figure 3*). It was then allowed to load fully for 5 minutes to allow the sample to completely absorb.

To extract the analytes from the SLE product, 2 x 500 µL of solvent (see *Table 1* for solvent used for each type of analyte) was added to each well and allowed to elute under gravity into a 1 mL collection plate (Cat no: 219250). Once both elutions were completed, 10 PSI of positive pressure was applied to the plate for 30 seconds to complete elution. The eluent was evaporated to dryness using $N_2$ with the Porvair Sciences Ultravap® Levante (Cat no: 500226) at 35°C and reconstituted in 200 µL of starting mobile phase as seen in the solvent composition sections of *Tables 1, 2* and *3*.

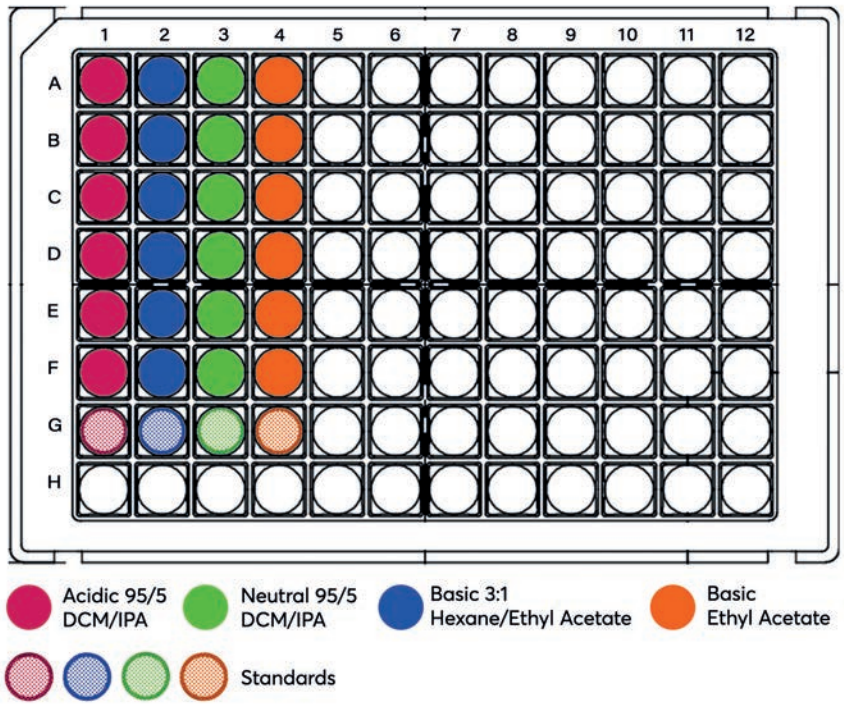



*Figure 3. Schematic of all plasma samples including matrix-matched standards (Row G) loaded onto the Microlute™ SLE 200 mg.*

**LLE**

LLE was performed using 1.5 mL centrifuge tubes. 2 x 500 µL of solvent (see *Table 1* for solvent used for each type of analyte) was added to 200 µL of diluted plasma (as with SLE, the same pre-treatment was applied). For each extraction, the samples were shaken for 5 minutes by hand and centrifuged for 5 minutes at 10,000 RCF. The organic layer was carefully transferred with a glass pasteur pipette from the tube into a collection plate (Cat no: 219250), evaporated to dryness with $N_2$ with the Porvair Sciences Ultravap® Levante (Cat no: 500226) at 35°C and reconstituted in 200 µL of starting mobile phase.

## Results and Discussion

**Chromatography**

*Figures 4, 5* and *6* represent chromatograms of basic, acidic and neutral analytes respectively. A Raptor Biphenyl (30 mm x 2.1 mm, 1.8 µm) column was used to separate the three classes of compounds with three different chromatographic methods (*Tables 1, 2, 3* and *4*) all in under 10 minutes.

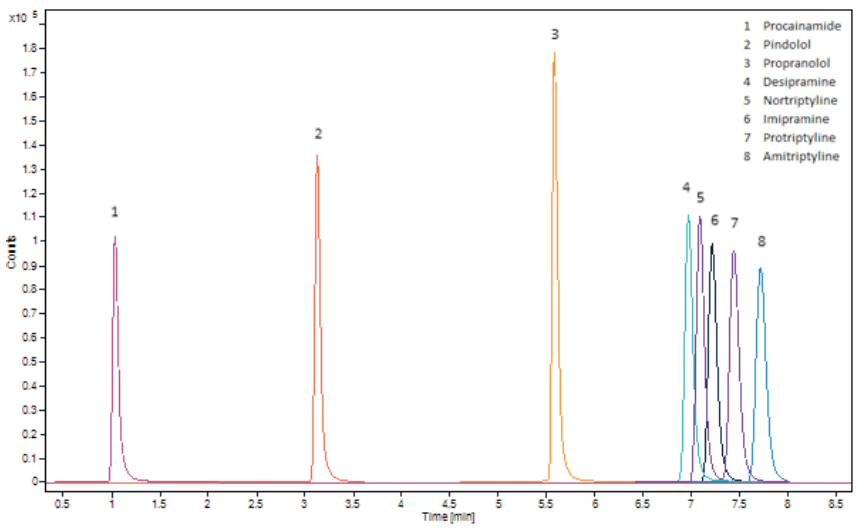



*Figure 4. Chromatogram of matrix match basic analytes standard at 0.5 µg/mL. Method is shown in Table 1 and SIM ion used is shown in Table 5.*

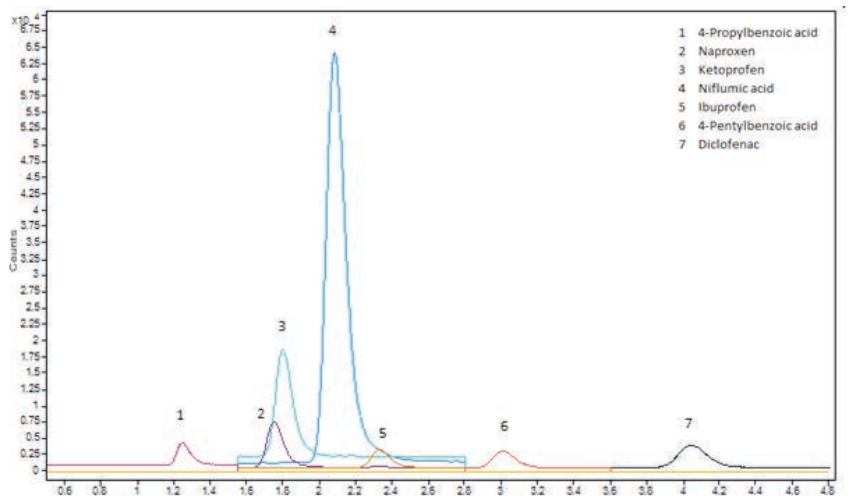



*Figure 5. Chromatogram of matrix match acidic analytes standard at 0.5 µg/mL. Method is shown in Table 2 and SIM ion used is shown in Table 5.*

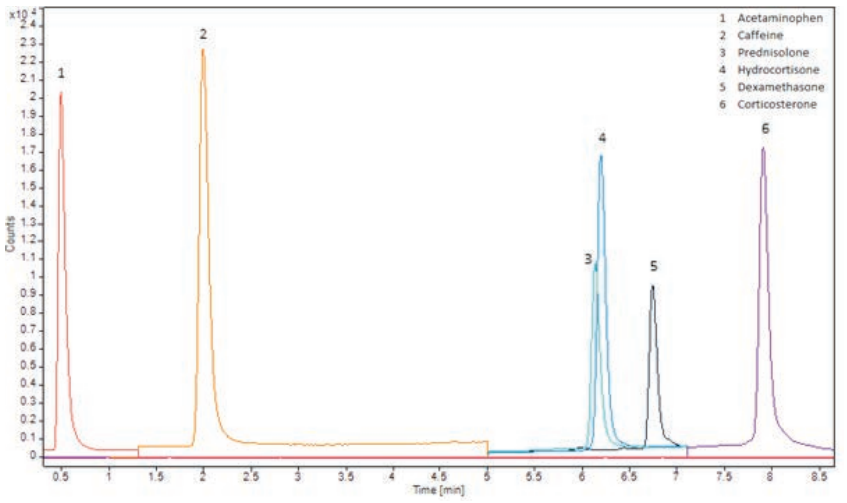



*Figure 6. Chromatogram of matrix match neutral analytes standard at 0.5 µg/mL. Method is shown in Table 3 and SIM ion used is shown in Table 5.*

## Recovery and Reproducibility

**Recovery and Reproducibility**

Analyte recovery was calculated using the following equation:

$$\%\ Recovery = \left(\frac{Area\ of\ Sample}{\text{Area of Matrix Match Standard}}\right) x\ 100\%$$

Analyte reproducibility was measured using relative standard deviation which was calculated using the following equation:

$$\%\ RSD = \left(\frac{Average\ Recovery\ of\ Compound}{\text{Standard Deviation of Recoveries}}\right) x\ 100\%$$

*Figure 7* shows the mean recoveries for SLE and LLE side by side. Microlute™ SLE shows improved reproducible recoveries across the analytes from each class (acidic, basic and neutral). The average recovery across all analytes tested was 94±6%. The recoveries ranged from 89 - 106%. For LLE recoveries were not as good with an average of 87±7% with recoveries ranging from 79-98%. So whilst it gave some high recoveries, on average it was lower than SLE and was less reproducible. For LLE recoveries can be improved by performing a third extraction on the sample. However, this would not provide a direct comparison to SLE and would further increase the sample processing time. It may also cause issues by introducing matrix effects, due to the further extraction of unwanted compounds which can affect the ionisation in the instrument's source.

When comparing the differences between the two techniques, SLE recovery is equivalent

or better for recovery than LLE. This was the case for every class of analyte - acidic, basic and neutral. This is due to the better extraction efficiency present in the Microlute™ SLE, offering a more effective solution to gain better recoveries in a shorter period with less repetitive processes in place. This is especially clear when looking at the more hydrophilic compounds (caffeine, procainamide and acetaminophen) which have a 10-20% increase in recovery in the SLE method.

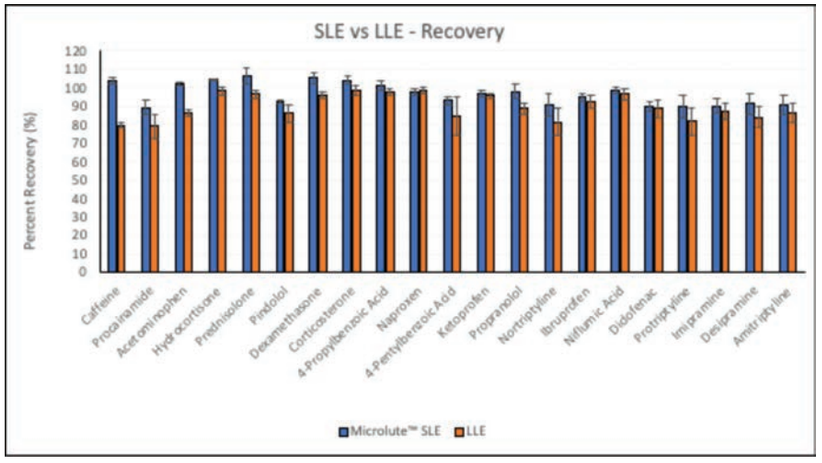



*Figure 7. Mean recoveries for all analytes in plasma samples using Microlute™ SLE 200mg and LLE (N=6). The error bars represent the standard deviations of the recovery results. Analytes are ordered in increasing Log P values.*

For reproducibility, results are shown in *Figure 8* which is represented as relative standard deviation (%RSD). The lower the %RSD value, the more reproducible the recovery is. Reproducibility is an important metric to allow confidence in results. If recovery is high but reproducibility is low, it can lead to questions on if the result was correct and could be acquired again. The average %RSD for the SLE recoveries was 3.2% which ranged from 0.7% to 6.9%. These values showed SLE was more reproducible than the LLE work which

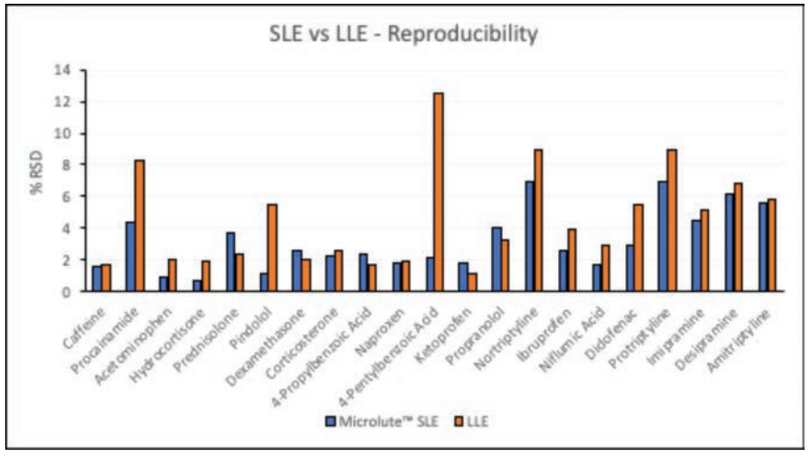
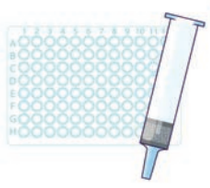



*Figure 8. Relative Standard Deviations (%RSD) for all analytes in plasma samples using Microlute™ SLE 200 mg and LLE (N=6). Analytes are ordered in increasing Log P values.*

averaged 4.8% and had a range from 1.2% to 12.5%. This difference has likely come from LLE having more steps which rely on the analyst's technique to ensure sample-to-sample extraction steps of reproducible – shaking and then extracting the organic layer.

## Processing time

Another area in which SLE outperforms LLE is the time to perform the sample preparation. *Figure 9* highlights the difference in processing times for both sample preparation methods. To prepare 96 plasma samples for SLE it is three times quicker when compared to the equivalent number of samples for LLE (40 minutes versus 129 minutes respectively). The biggest time saving element arises from the lack of labour-intensive steps of sample shaking and transfer of organic solvent layers in LLE.



| SLE Workflow – 40 Minutes | |
|---|---|
| **SLE Steps** | **Time (min)** |
| Pipette sample | 6 |
| Adsorb | 5 |
| Solvent extractions (2) | 16 |
| Evaporate | 10 |
| Reconstitute | 3 |
| **Total Time** | **40 min** |

| LLE Workflow - 129 Minutes | |
|---|---|
| **LLE Steps** | **Time (min)** |
| Pipette sample | 12 |
| Add solvent | 12 |
| Shake | 5 |
| Centrifuge | 5 |
| Transfer extracts | 30 |
| Add solvent | 12 |
| Shake | 5 |
| Centrifuge | 5 |
| Transfer extracts | 30 |
| Dry down | 10 |
| Reconstitute | 3 |
| **Total Time** | **129** |

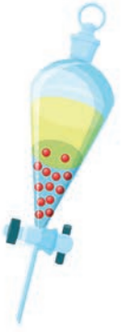


*Figure 9. Protocols and the processing times for each step of the LLE and SLE procedures for 96 samples.*

## Conclusion

This application note compared SLE and LLE methods for recovery, reproducibility and methodology using pig plasma on a range of compounds from acidic, basic and neutral classes. The comparison of the two methods demonstrated that the Microlute™ SLE plate is superior both in performance (able to obtain higher and more reproducible results) and the time taken to complete the sample preparation, compared to the equivalent LLE method performed. Using Porvair Sciences Microlute™ SLE plate allows greater sensitivity and more confidence in the results. With the ability for high throughput by reducing the labour-intensive steps associated with LLE it should be the sample preparation method of choice for any analyst.

Related Products: Microlute™ SLE 200 mg, 3 ml cartridge (PSLE2003-050), Microlute™ SLE 400 mg, 3 ml cartridge/plates (PSLE4003-050/ PSLE400P-001), 1.1 ml 96-well Low Profile Collection Plate (#219250), 2.2 ml, 96 well, V-bottom Collection Plate (#219009).

## References:

*1. Ramirez C, Peters K. Extraction Techniques for Food Processing. ED-Tech Press; 2018.*