



Extraction of Methadone & Imipramine from Serum Using Immobilized Liquid Extraction™ (ILE) Well Plates

Abstract

The following application note demonstrates the ability of Immobilized Liquid Extraction Well Plates to provide high-throughput bioanalytical extractions of methadone and imipramine directly from serum. ILE Well Plates exhibit highly efficient (99.6%) and reproducible ($\pm 0.6 - 4.1\%$ RSD) extractions for a wide range of analyte concentrations (10 – 20,000 ng/mL).

Introduction

Immobilized Liquid Extraction (ILE) Well Plates provide analysts reliable, rapid and environmentally friendly high-throughput extractions without protein crashing, emulsion formation, solvent exchange or dry wells. ILE Well Plates involve minimal user management, especially on a well-to-well basis, do not require vacuum or pressure systems, and are capable of extracting small molecules directly from blood, serum or any aqueous biological matrix.

Traditionally, extractions of small molecules from biological fluid samples (plasma, serum, urine, etc.) have utilized liquid-liquid extraction (LLE), or liquid-solid extraction employing Solid Phase Extraction (SPE) disks or cartridges prior to LC-MS or GC-MS analysis.

Each of these techniques is plagued by serious shortcomings. LLE is labor intensive, vulnerable to the formation of emulsions, and may require protein crashing or solvent exchange. SPE involves a complex multi-step procedure which is prone to error caused by analyte breakthrough or clogged SPE disks/cartridges. Both techniques involve excessive quantities of solvent, and often require a solvent evaporation, concentration, or exchange step. Additionally, neither LLE nor SPE allows for easy or efficient automation, and therefore both are poorly suited to high-throughput bioanalytical applications.

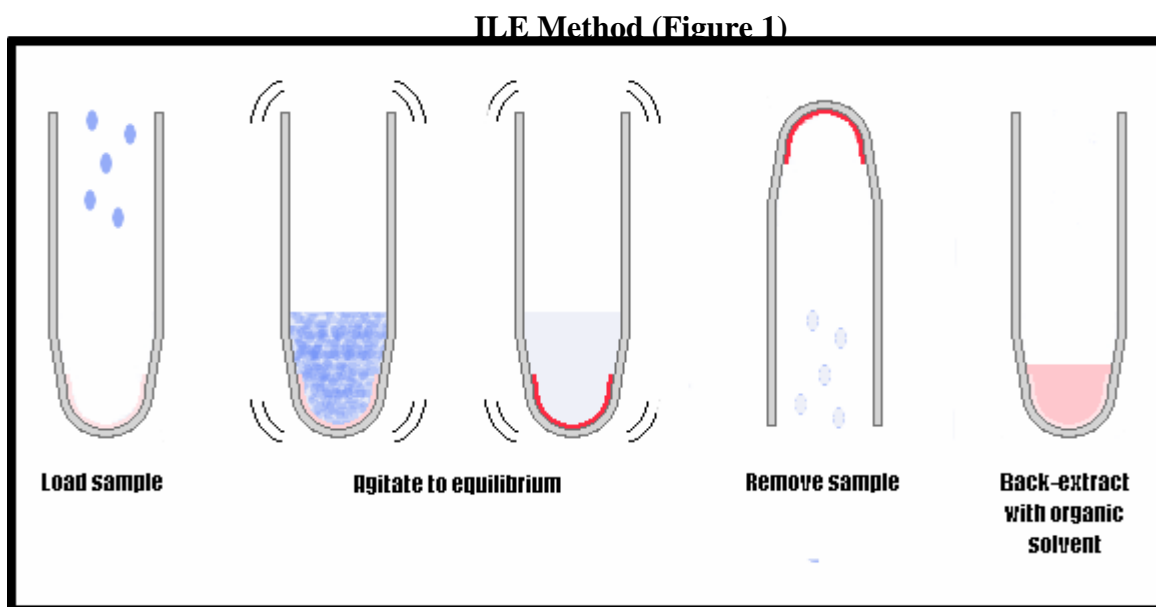
This application note describes the development of an easily automated procedure for high-throughput extractions of drugs from serum using ILE 96 Well Plates. Analyte recovery, speed, efficiency and reproducibility are evaluated.

ILE Methodology

Each well of an ILE Well Plate is coated with a thin layer of polymer (immobilized liquid) which acts as the medium of extraction. When a sample is directly exposed to the polymer, targeted analytes partition between the aqueous sample and the immobilized phase at a predictable rate. This partitioning process is allowed to occur until extraction equilibrium is attained. The quantity of a specific analyte that is extracted by the immobilized phase is determined both by the analyte's relative affinity for both the sample and the immobilized phase (*partition ratio*), as well

as by the relative volumes of the sample and the extracting polymer (*phase ratio*). For example, analytes ultimately partition between an aqueous sample and a polydimethylsiloxane (PDMS) phase at a rate similar to that between water and octanol (i.e., $K_{PDMS/W} \approx K_{O/W}$).

The partitioning process may be accelerated by agitating the ILE Well Plate with a vortexer, sonicator or another appropriate method. Proper agitation disrupts a boundary layer which forms at the sample-polymer interface, a consequence which may alternatively be accomplished by repeatedly dispensing and aspirating a sample into and out of each well. Analytes which partition into the polymer are 'back-extracted', or desorped, into a small amount of LC or GC solvent, at which point the extraction is complete. The entire ILE process is displayed in Figure 1.



Automating ILE

ILE Well Plate extractions may easily be performed manually or in conjunction with an automated liquid handling system. Unlike SPE Well Plates or (semi-)automated LLE methods, ILE Well Plates require minimal user management, especially on a well-to-well basis. SPE well plates, utilize a flow-through system which requires sorbent conditioning and a source of vacuum or pressure. Because the sorbent bed of each SPE well is never packed exactly the same, flow rates may fluctuate from one well to the next, potentially resulting in dry wells. Further, SPE wells are inherently prone to being clogged by samples which are viscous or contain large quantities of particulate. Similarly, high-throughput LLE requires operator attention on a well-to-well basis due to the possibility of emulsion formation. An additional centrifugation step may also be required in small-scale LLE extractions to separate an extracting solvent from a sample.

ILE Well Plates are immune to clogging and the formation of emulsions, and require neither vacuum/pressure systems nor protein precipitation. Derivatizing reagents have no adverse effect on the polymer; therefore back-extraction and derivatization may be performed simultaneously. Little to no human attention or interaction is required to use ILE Well Plates, especially on a

well-to-well basis, and hence ILE Well Plates are a very practical and efficient method for high-throughput bioanalytical applications.

Experimental Details

Samples: All samples consisted of 100µl goat serum (Midland Bio Products) diluted 1:1 with water, and adjusted to pH 11 with 10M NaOH. Samples and non-extracted calibration spikes were created appropriately at concentrations described herein using certified analytical standards.

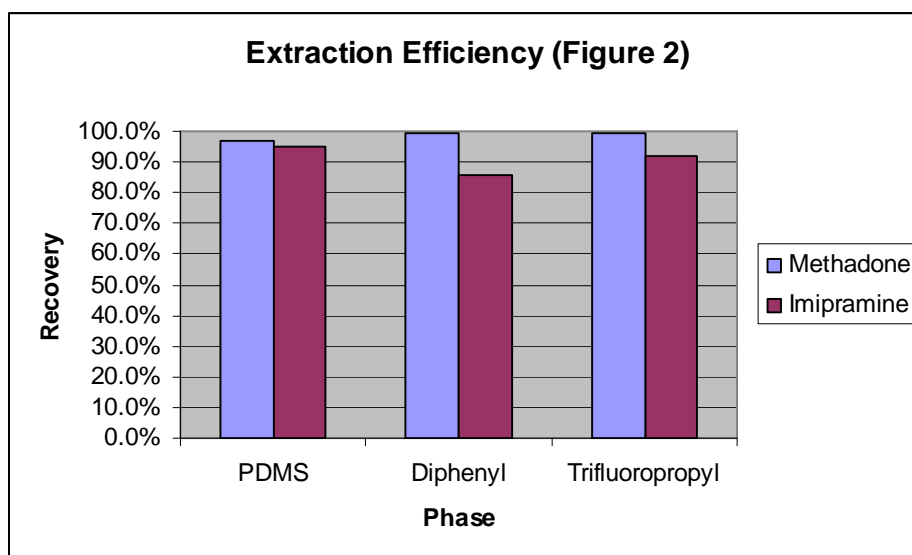
Back-Extraction Solvent: J.T. Baker Baker Analyzed® Acetonitrile (HPLC Grade)

GC-MS Conditions: HP-5971 GC-MS in SIM mode, 1µl splitless injection valve, on a 30m x 0.25mm i.d. X 0.25µm VB-5 column.

Results

Extraction Efficiency:

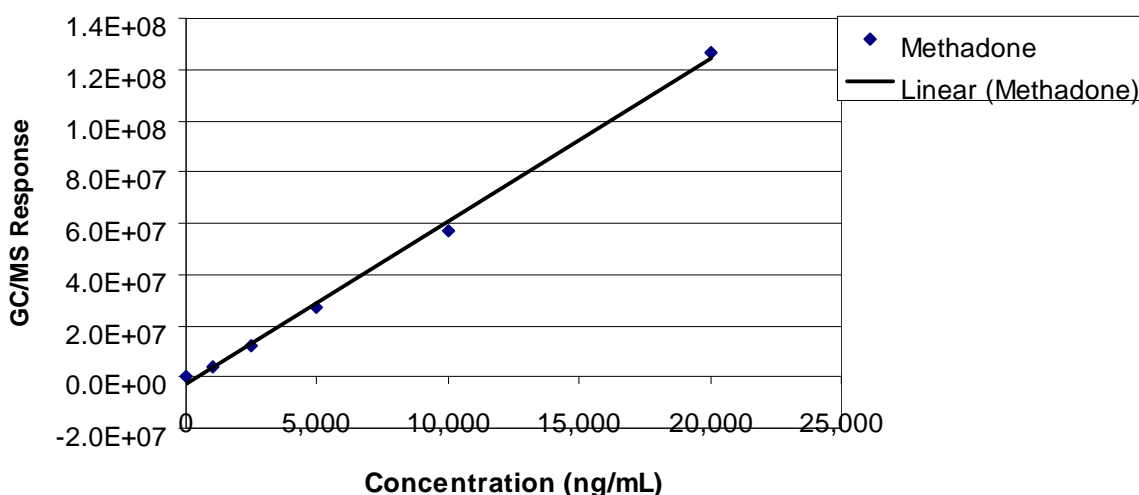
Extraction efficiency was investigated by extracting known quantities (1µg/mL) of methadone and imipramine from 100µL goat serum samples. The MS response given for each extracted sample was compared to the MS response of an unextracted standard that represents 100% recovery. Recoveries were measured for three extracting phases: Polydimethylsiloxane (PDMS), polydiphenylsiloxane (diphenyl), and trifluoropropylmethylsiloxane (fluoro). Recoveries ranged from 86.0 to 99.6 percent with relative standard deviations from 0.6 to 4.1 percent (**Figure 2**).



Standard Curve:

A series of experiments was performed to show a linear correlation between sample concentration and MS response at a range of concentrations. 100µL goat serum samples were diluted 1:1 with water and adjusted to pH 11 with 10M NaOH. Each sample was spiked with a known concentration of methadone. Three replicate samples were prepared at each of the following six concentrations: 10ng/mL, 1,000ng/mL, 2,500ng/mL, 5,000ng/mL, 10,000ng/mL and 20,000ng/mL. Each sample was extracted using a trifluoropropyl phase and the ILE method. Namely, samples were placed in an ILE Well Plate, and the plate was sealed and agitated by a vortexer for 45 minutes at 3,000 RPM. The samples were removed from the plate, and 150µl of acetonitrile was dispensed into each well. The ILE Well Plate was sealed again and placed on a vortexer for 20 minutes at 3,000 RPM to ensure that analytes sufficiently back-extracted into the acetonitrile. Sample extracts were analyzed by GC-MS in SIM mode. The results are depicted below (Figure 3).

Standard Curve (Figure 3)



Extraction and Back-Extraction Times

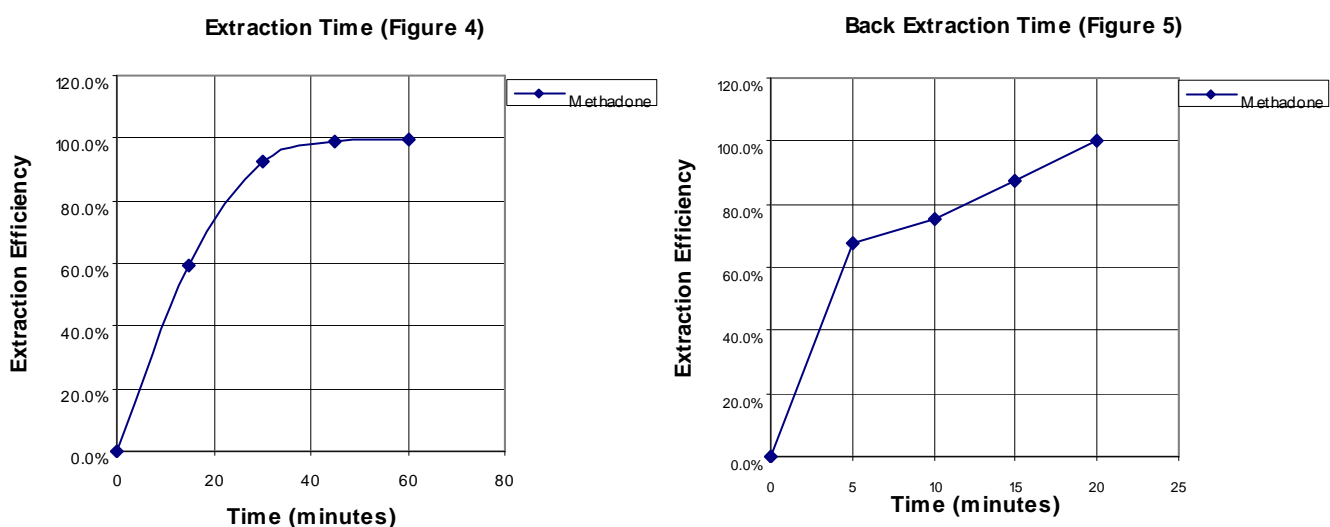
Extraction and back-extraction times were optimized for ILE Well Plate extractions of methadone from serum which were assisted by a Scientific Industries Microplate Genie™ vortexer operating at 3,000 RPM. Extraction and back-extraction times may vary for different compounds, sample matrices, or sources of agitation. Determinations of optimal extraction and back-extraction times were made by extracting a series of samples spiked with methadone at a concentration of 1.2µg/ml.

The determination of optimal extraction time consisted of comparing the MS response for a series of samples which were extracted for 15, 30, 45 and 60 minutes, respectively. To independently determine the optimal extraction time for ILE Well Plates, each extracted sample was back-extracted for a period of time that ensured complete desorption of analytes into the

acetonitrile – in this case 30 minutes. Experimental data reveals an optimal extraction time of 45 minutes, where analyte recovery of 99.1% is achieved. This is depicted in Figure 4, which also shows that if extraction time were reduced to 30 minutes, 92% extraction efficiency is still attained.

Optimal back-extraction time was determined using a similar method. A series of samples was extracted for 45 minutes and back-extracted with 150µl acetonitrile for 5, 10, 15 and 20 minutes, respectively. A back-extraction time of 20 minutes was determined to be optimal (Figure 5).

It should be noted that **complete** back-extraction is recommended for a few reasons. First, if back-extraction is not brought to equilibrium, there will be drastic variability in results on a well-to-well basis. And second, the back-extraction process is very rapid, so ensuring complete back-extraction will have a negligible effect on throughput.



Conclusions

Immobilized Liquid Extraction is a promising technique for extracting small molecules directly from biological matrices that avoids many of the shortcomings of traditional techniques. ILE Well Plates have been experimentally demonstrated to provide highly precise ($\pm 0.6 - 4.1\%$) and efficient (86.0 – 99.6%) extractions. A linear correlation between sample concentration and MS response was defined across a wide range of analyte concentrations (10 – 20,000 ng/mL). Little to no human attention or interaction is required to use ILE Well Plates, especially on a well-to-well basis. Solvent usage, and subsequent disposal, is minimized. Consequently, ILE Well Plates provide a practical, cheap and efficient solution for high-throughput bioanalytical applications.