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Automating Liquid-Liquid Extractions using a Bench-top Workstation

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Abstract
Liquid-liquid extractions have long been performed manually and are used to extract and concentrate analytes from aqueous matrices. Inclusion of liquid-liquid extraction in many official methods attests to the wide acceptance of the technique. Following solvent extraction it is also common to include an evaporation and reconstitution step to improve detection limits or exchange solvents for compatibility with subsequent chromatographic separations. Modern analytical labs are looking to automation to help reduce solvent usage and increase sample throughput while ensuring the high quality of the resulting data.

An X-Y-Z coordinate autosampler, the GERSTEL MultiPurpose Sampler (MPS), commonly used for sample introduction in GC or HPLC can be used to perform a wide variety of sample preparation techniques using a single instrument and controlling software. The sampler can be configured as part of a GC or LC system or can be configured as a bench-top workstation and can also include a six position evaporation station.

In this report, the automation of liquid-liquid extractions using the MPS autosampler is discussed. Examination of a new, automated vortexing option that allows samples to be rapidly and effectively mixed using speeds of up to 3000 rpm is described. Automated liquid-liquid extractions methods for a variety of analytes from different matrices are examined and resulting precision and accuracy data are provided.

INTRODUCTION

Liquid-liquid extraction is a fundamental sample preparation technique used in chemical laboratories to extract analytes from a variety of different matrices. During such solvent extractions, a partitioning method is used to separate compounds based on their relative solubility in two different immiscible liquids (e.g., water and an organic solvent). As a general rule, liquid-liquid extractions are performed manually and are quite labor intensive.

Automation is known to improve the precision of liquid transfers. A dual head version of the MultiPurpose Sampler (MPS XL) allows two different syringes to be used without having to spend time changing syringes between different steps in the workflow. This means that a wider range of volumes can be transferred automatically in one workflow enabling automation even of complex manual liquid-liquid extractions and thereby minimizing laboratory staff exposure to potentially hazardous organic solvents. Furthermore, rapid vortexing of organic solvents with sample matrices provides an alternative to traditional shaking methods and may allow for a higher throughput of such extraction procedures.

The aim of this study was to show that a manual liquid-liquid extraction procedure can easily be translated into an automated Prep Sequence performed by the MPS Dual Head WorkStation under MAESTRO software control.

Automation of the method development of liquid-liquid extraction methods can also be performed using the same MPS platform. With additional modules, the injection of the final extract can be controlled, which would allow the analyst to perform automated method development followed by analytical detection. Using such a strategy, the analyst can start the system before leaving for the day and obtain data that would identify an optimized extraction procedure by the time he or she comes in the next morning.

EXPERIMENTAL

Materials. All analyte stock solutions were purchased from Cerilliant. Intermediate analyte stock solutions were prepared by combining the appropriate analyte stock solutions with methanol, at appropriate concentrations, to evaluate the different analytes. Final standards for calculating % Recoveries were prepared by combining the appropriate analyte stock with (90:10) 0.05 % formic acid in water:acetonitrile. Deuterated analogues, d_4-ketamine, d_3-buprenorphine, and d_3-norbuprenorphine, were purchased from Cerilliant. A working internal standard stock solution containing the d_4-ketamine internal standard was prepared at a concentration of 5000 ng/mL in methanol. A working internal standard stock solution containing the d_3-buprenorphine and d_3-norbuprenorphine internal standards was prepared at a concentration of 5000 ng/mL of each, in methanol.

Horse serum (cat.#H0146-10ML) was purchased from Sigma-Aldrich. Ketamine spiked horse serum samples were prepared by making appropriate dilutions of the ketamine intermediate analyte stock solution using analyte free horse serum to give a final concentration of 500 ng/mL.

Lyophilized bovine plasma (cat.#P4639) was purchased from Sigma-Aldrich and was reconstituted as directed using 10 mL of LCMS grade water. Buprenorphine and Norbuprenorphine spiked bovine plasma samples were prepared by making appropriate dilutions of the buprenorphine/norbuprenorphine intermediate analytes stock solution using analyte free bovine plasma to give a final concentration of 500 ng/mL of each analyte.

All other reagents and solvents used were reagent grade.

Instrumentation. All automated Prep Sequences were performed using a bench top, dual-head MPS WorkStation configured with GERSTEL mVORX, mVAP, and Universal Filtration Options as shown in Figure 1.

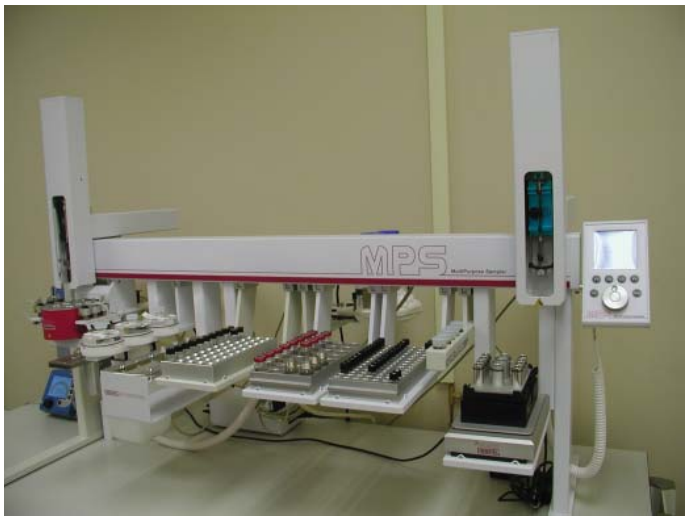

Figure 1. GERSTEL MultiPurpose Sampler (MPS) Dual Head WorkStation configured with mVORX, mVAP, and Universal Filtration Options.

All analyses were performed using an Agilent 1290 HPLC with a Poroshell 120, EC-C18, (3.0 x 50 mm, 2.7 µm), an Agilent 6460 Triple Quadrupole Mass Spectrometer with Jet stream electrospray source and a GERSTEL MPS XL configured with an Active Washstation. Sample injections were made using a 6 port (0.25 mm) Cheminert C2V injection valve outfitted with a 2 µL stainless steel sample loop.

A liquid-liquid extraction procedure for ketamine from biological matrices was adapted with slight modifications found in literature1; the procedure included the following steps.

Ketamine Liquid-Liquid Extraction Procedure:

1. 1 mL of serum sample is placed into a 10 mL vial, which is sealed with a magnetic cap and placed in the autosampler tray.
2. Add 100 µL of 5000 ng/mL working internal standard to the sample.
3. Add 1 mL of 10 mM potassium hydroxide to the sample.
4. Add 5 mL of (7:3) methyl-t-butyl ether: dichloromethane to the sample.
5. Vortex for 5 minutes at 2000 rpm.
6. Centrifuge samples for 5 minutes at 3000 rpm.
7. Transfer 4 mL of the organic layer to a clean, 10 mL vial that has been capped with a magnetic cap.
8. Evaporate to dryness at 40°C for 20 minutes.
9. Reconstitute residues using 0.500 mL of (90:10) 0.05 % formic acid in water: methanol.
10. Filter resulting sample using a 0.45µm nylon filter into a clean 2 mL autosampler vial.
11. Inject 2 µL into the LC/MS/MS system.

Figure 2 shows the MAESTRO Prep Sequence used to perform the steps listed above in a fully automated manner.

Action	MPS	Method / Value	Source	Vial	Destination	Vial
PREP Vials 1		Ahead, Extensive				
ADD	Right MPS	Add 100uL of WIS to samples	Tray3,VT98	98	Tray1,VT32-10	
WASH	Right MPS	Wash syringe after WIS transfer				
ADD	Left MPS	Add 1mL 10mM KOH to sample	Tray1,VT32-10	32	Tray1,VT32-10	
WASH	Left MPS	Wash syringe x2 using IPA				
ADD	Left MPS	Add 5mL MTBE_MeCl2 to sample	Tray1,VT32-10	+28	Tray1,VT32-10	
WASH	Left MPS	Wash syringe x2 using IPA				
MOVE	Right MPS		Tray1,VT32-10		mVorx,mVTC1-10	
MIX	Right MPS	Vortex sample for 5min at 2000rpm				
MOVE	Right MPS		mVorx,mVTC1-10		Tray1,VT32-10	
WAIT	Left MPS	Prompt user to perform offline centrifugation				
ADD	Left MPS	Transfer 4mL Extraction Solvent	Tray1,VT32-10		Tray2,VT32-10	
MVAP EVAPORATE	Left MPS	Evaporate MTBE_MeCl2 at 40C for 20min	Tray2,VT32-10		Tray2,VT32-10	
WAIT	Left MPS	Prompt user to inspect vials following evaporation				
ADD	Left MPS	Recon using 500uL recon solution	Tray2,VT32-10	24	Tray2,VT32-10	
MOVE	Left MPS		Tray2,VT32-10		mVap,mVapTray	
MIX	Left MPS	Mix samples after recon for 30sec				
MOVE	Left MPS		mVap,mVapTray		Tray2,VT32-10	
MOVE	Left MPS		Tray3,VT98		FLTSampl,VTFLTV	
FILTER	Left MPS	GET	FilterT1,FT12-FLT			
FILTRATE	Left MPS	Filter reconstituted sample into 2mL vial	Tray2,VT32-10		FLTElute,ET1-FLT	
END						

Figure 2. Automated Prep Sequence used for Ketamine Liquid-Liquid Extraction.

Automated Liquid-Liquid Extraction Method Development. In order to automate the method development of a liquid-liquid extraction procedure for Buprenorphine and Norbuprenorphine in bovine plasma, solvent reservoirs containing the following solvents and pH adjustment solutions were placed onto the dual-head MPS XL:

Organic Solvent	*Polarity Index*
(99:1) Hexane: IPA	0
Methyl-t-butyl ether	2.5
Methylene Chloride	3.1
Ethyl Acetate	4.4

pH Adjustment Solutions	*pH*
10 mM Hydrochloric acid	acid
10 mM Ammonium Acetate	neutral
10 mM Potassium Hydroxide	base

Analytical Method LC Method Parameters.

Mobile Phase:	A - 5 mM ammonium formate with 0.05 % formic acid
	B - 0.05 % formic acid in methanol
Gradient:	Initial 5 % B
	0.5 min 5 % B
	1.5 min 30 % B
	3.5 min 70 % B
	4.5 min 95 % B
	6.5 min 95 % B
	7.5 min 5 % B
Pressure:	600 bar
Flowrate:	0.5 mL/min
Run time:	6.5 min
Injection volume:	2 µL (loop over-fill technique)
Column Temp.:	55°C

Analytical Method Mass Spectrometer Parameters.

Electrospray positive mode + Agilent Jet Stream

Gas Temperature:	350°C
Gas Flow (N2):	5 L/min
Nebulizer pressure:	35 psi
Sheath Gas Temp:	250°C
Sheath Gas Flow:	11 L/min
Capillary voltage:	4000 V
Nozzle voltage:	500 V

The mass spectrometer acquisition parameters for Ketamine are shown in Table 1 along with the qualifier ion transitions. The mass spectrometer acquisition parameters for Buprenorphine and Norbuprenorphine are shown in Table 2 along with the qualifier ion transitions. A retention time window value of 1.25 minutes was used for each positive ion transition being monitored during the course of each of the two dynamic MRM experiments.

Table 1. Ketamine Mass Spectrometer Acquisition Parameters

Compound	Precursor Ion [m/z]	Product Ion [m/z]	Frag. [V]	CE [V]	Ret. Time [min]
D_4-Ketamine	242.1	129	102	32	2.466
	242.1	119	102	68	2.466
Ketamine	238.1	220.1	105	11	2.474
	238.1	125	105	11	2.474

Table 2. Buprenorphine/Norbuprenorphine Mass Spectrometer Acquisition Parameters

Compound	Precursor Ion [m/z]	Product Ion [m/z]	Frag. [V]	CE [V]	Ret. Time [min]
Buprenorphine	468.3	396.2	200	41	3.546
	468.3	55.1	200	60	3.546
D_3-Norbuprenorphine	417.3	152	190	124	3.05
	417.3	55.1	190	76	3.05
D_4-Buprenorphine	472.3	400.2	210	44	3.511
	472.3	59.1	210	60	3.511
Norbuprenorphine	414.3	187.1	205	41	3.06
	414.3	83.1	205	57	3.06

Results and Discussion

The starting concentrations of the spiked horse serum and bovine plasma samples were chosen so that after being extracted and reconstituted they should have the same theoretical concentrations as their respective neat standards. In this way, direct comparisons could be made to assess the % Recovery of the automated liquid-liquid extraction procedures.

As shown in Table 3, the % Recovery of Ketamine following automated liquid-liquid extraction was found to be 88.7 % based on the resulting response ratio values. The precision of the automated liquid-liquid extraction for Ketamine was also assessed using the four individual horse serum replicates extracted using the automated method. Precision data was found to be 0.467 % CV using the resulting response ratio values. These data show that a manual liquid-liquid extraction procedure is easily translated into a Prep Sequence using the MAESTRO software and automated using the Dual Head MPS XL, delivering excellent quality results.

Table 3. Results of Automated Ketamine Liquid-Liquid Extraction

Sample Name	Response	ISTD Response	Response Ratio
1000 ng/mL neat Ketamine std 1	11878597	16748195	0.7092
1000 ng/mL neat Ketamine std 2	12544625	17637186	0.7113
1000 ng/mL neat Ketamine std 3	12259205	17294488	0.7089
mean	12227475	17226623	0.7098
SD	334146	448364	0.00129
% CV	2.73	2.60	0.182

Sample Name	Response	ISTD Response	Response Ratio
LiqLiq Extr rep 1	9794014	15573317	0.6289
LiqLiq Extr rep 2	9891262	15714821	0.6294
LiqLiq Extr rep 3	9484299	15152067	0.6259
LiqLiq Extr rep 4	9761104	15417946	0.6331
mean	9732670	15464538	0.6293
SD	174558	241028	0.00294
% CV	1.79	1.56	0.467
% Recovery	79.6	89.8	88.7

A general rule of thumb when performing liquid-liquid extractions is that a greater number of extractions with smaller volumes will extract greater quantities of analytes compared with a single extraction using a larger volume of extraction solvent. Since we were aiming to demonstrate that automated method development for an unknown analyte could be performed, during the course of the automated method development for Buprenorphine and Norbuprenorphine from bovine plasma, multiple extractions using smaller extraction solvent volumes were performed as part of this process. Automated liquid-liquid extractions using such a strategy are set up in a very simple manner - by mouse-click - using the MAESTRO software.

The MAESTRO software also allows the analyst to pause a running Prep Sequence, which can be especially important for automated methods that require an offline step to be performed. In order to demonstrate this, as an example, the PROMPT command was used during the automated method development Prep Sequence. In this way, the Prep Sequence could be paused, allowing for offline centrifugation of samples prior to transfer of the organic layers. Figure 3 shows the graphical analyst interface window that is displayed during the course of the Prep Sequence, prompting the analyst when it is time to take the samples off the sampler, centrifuge at a specific speed, and then place the samples back into their original positions in order to continue with the automated method.

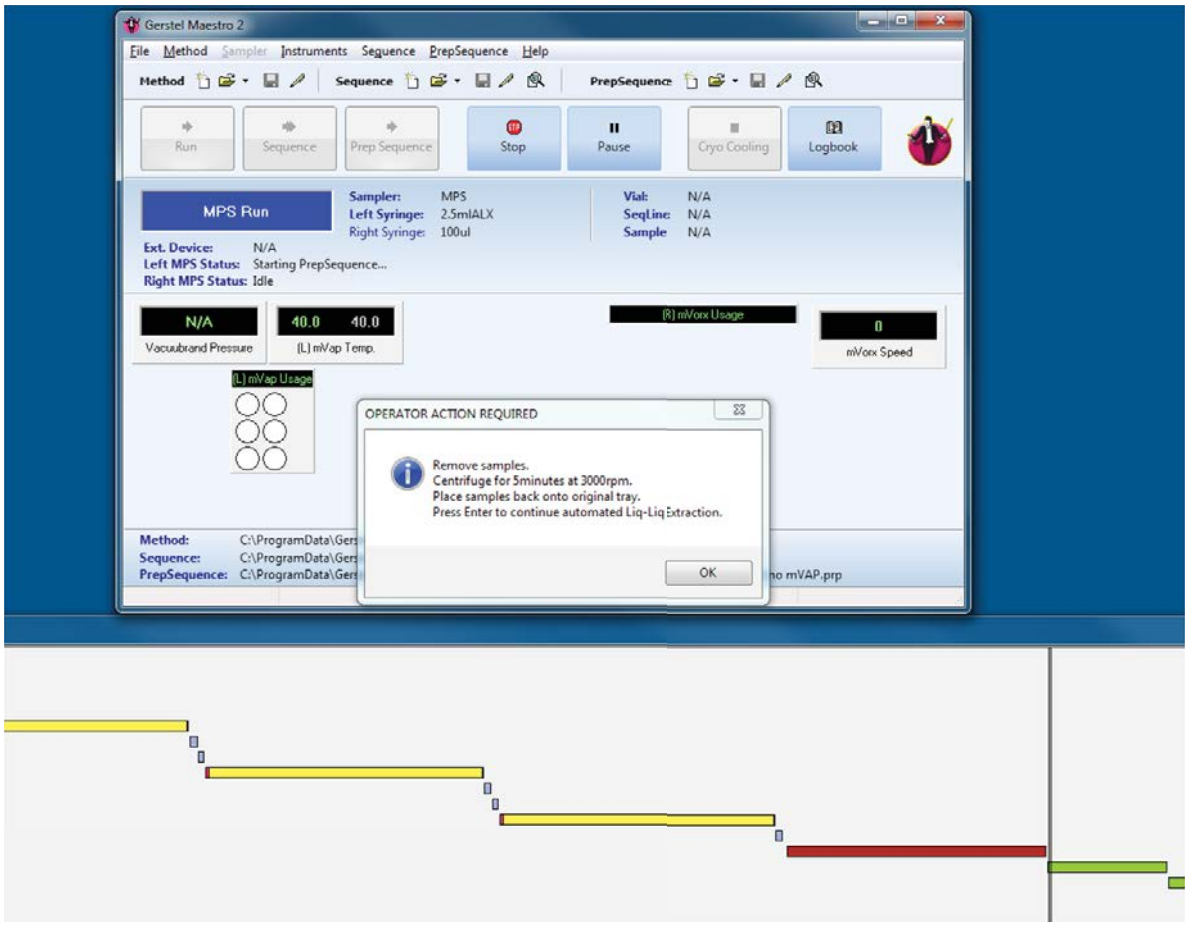

Figure 3. MAESTRO software "PROMPT" command.

The extracts generated over the course of the automated liquid-liquid extraction method development sequence were subsequently analyzed. The analysis results obtained from the extracted samples were compared with those from their respective neat standards. This allowed us to assess which combination of extraction solvent and pH adjustment solution yielded the best extraction efficiency by comparing the respective %Recoveries for each analyte. As shown in Figure 4, the (99:1) Hexane: IPA extraction solvent resulted in the best %Recovery for both Buprenorphine and Norbuprenorphine under acidic conditions.

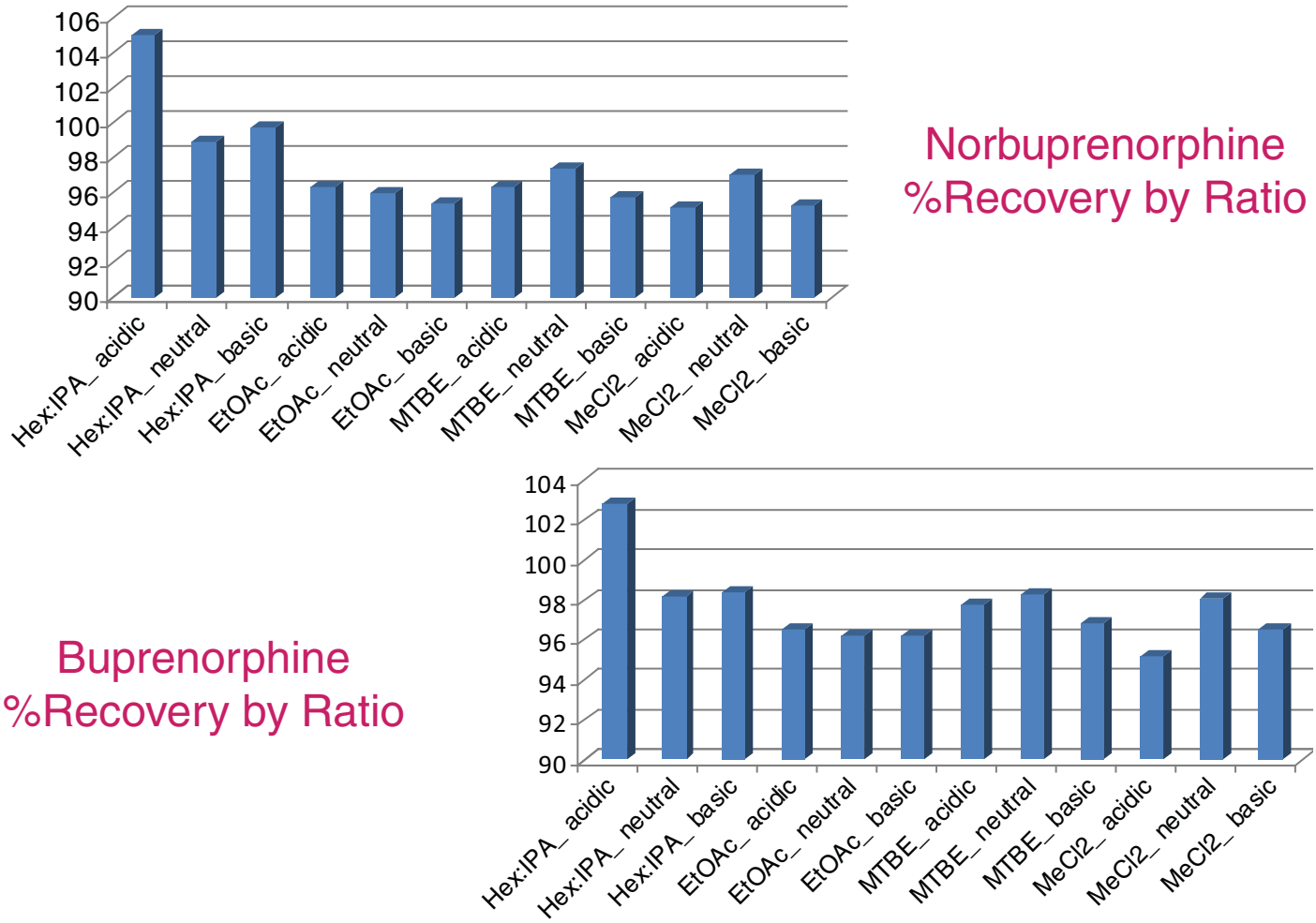

Figure 4. Assessment of Automated Liquid-Liquid Extraction Method Development using Resulting % Recoveries.

Conclusions

As a result of this study, we were able to demonstrate:

- Manual liquid-liquid extraction procedures can easily be translated into automated Prep Sequences using the MAESTRO software.
- Precision data from the automated liquid-liquid extraction method was found to be 0.467 % CV using the resulting response ratio values.
- The method development of liquid-liquid extraction methods can be automated using the MAESTRO software and the Dual Head MultiPurpose Sampler (MPS XL).

References

[1] S. Bolze and R. Boulieu, "HPLC Determination of ketamine, norketamine, and dehydronorketamine in plasma with high-purity reversed-phase sorbent." Clinical Chemistry 1998, 44:3, 560-564.

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