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Application Note

Automated Bioanalytical SPE Method Development & Optimization for Oasis HLB µElution Using the 20 Bottle Approach

Nikunj Tanna, Mary Trudeau

Waters Corporation

Abstract

Solid phase extraction (SPE) is routinely used sample preparation technique in bioanalytical/DMPK laboratories, selectively capturing the analyte or drug target, while removing biomatrix components, thus improving the sensitivity, selectivity, and robustness of the analytical method. SPE is often perceived as complex and labour-intensive, requiring lengthy method optimization to achieve high analyte recovery. Here, we highlight an elegant, simple, and broadly applicable, fully automated approach for fast reversed-phase SPE method optimization that using Andrew+ pipetting robot with Extraction+ and the Oasis® HLB reversed-phase sorbent in the 96-well format, which significantly simplifies SPE method optimization.

Benefits

- Elegant, fast and simple approach Oasis HLB SPE protocol optimization
- High analyte recovery >75%, with excellent reproducibility
- Automated SPE method development using Andrew+ Pipetting Robot and Extraction+
- Easy, one click, download & use OneLab library method for SPE optimization

Introduction

Bioanalysis, or the analysis of drugs and their metabolites from biological fluids, is used in support of pharmaceutical drug discovery, development, and research. When optimizing an analytical method for the analysis of a target analyte in wide range of matrices, scientists need to focus on three major aspects: 1-extraction of analyte/s of interest from matrix, 2-separation of analyte/s of interest form other matrix components, and 3-detection. Common sample preparation techniques for bioanalysis include dilution, protein precipitation (PPT), liquid-liquid extraction (LLE), and solid phase extraction (SPE). SPE is often used in bioanalytical/DMPK laboratories as a sample preparation technique to improve extraction recoveries, obtain cleaner samples as well as to concentrate samples without the need for evaporation (which may cause sample loss). This usually results in an improvement in sensitivity, selectivity, and robustness of the analytical method.

Regardless of the technique, sample preparation can be time consuming and complex, and is often perceived as the "bottle-neck" in the analytical workflow, with the need to develop and optimize every step of the extraction protocol. For SPE, the critical steps of the workflow that can be optimized include the wash and elution solvent. The Oasis HLB care & use manual (715000109 < https://www.waters.com/webassets/cms/support/docs/715000109.pdf>) provides a starting protocol that provides reasonable recoveries for a diversity of analytes. Additionally, it also describes an elegant SPE experimental design (Figure 1) which allows scientists to optimize the composition of the wash and elution solutions in a single experiment, thereby simplifying Oasis HLB method optimization.

Another key aspect critical to assays in bioanalytical labs is reproducibility. Intra and inter-day precision and accuracy are key metrics used during method validation to ensure the assay is fit-for-use. Automation and liquid handling robots are increasingly being deployed in these labs to improve reproducibility of assays, as well as unlock scientist's time to focus on more challenging scientific problems. Andrew+ pipetting robot with Extraction+, alongside OneLab™ informatics platform provides scientists with a simple, flexible, and easy to implement solution for their lab liquid handling/automation needs.

This work demonstrates the 20-bottle method optimization approach, which allows scientists to finetune the 2 most critical SPE steps (wash and elution) in a single experiment. Here, we have used Andrew+ Pipetting Robot configured with the Extraction+ connected device and created a OneLab library method to perform the 20 Bottle SPE method optimization using Oasis HLB SPE in the 96-well µelution plate format as described in the care and use manual. This OneLab method is ready to be downloaded and used by scientists from the OneLab library without the need for any modifications, offering an automated, fast, simple, and efficient way to perform SPE

method optimization.

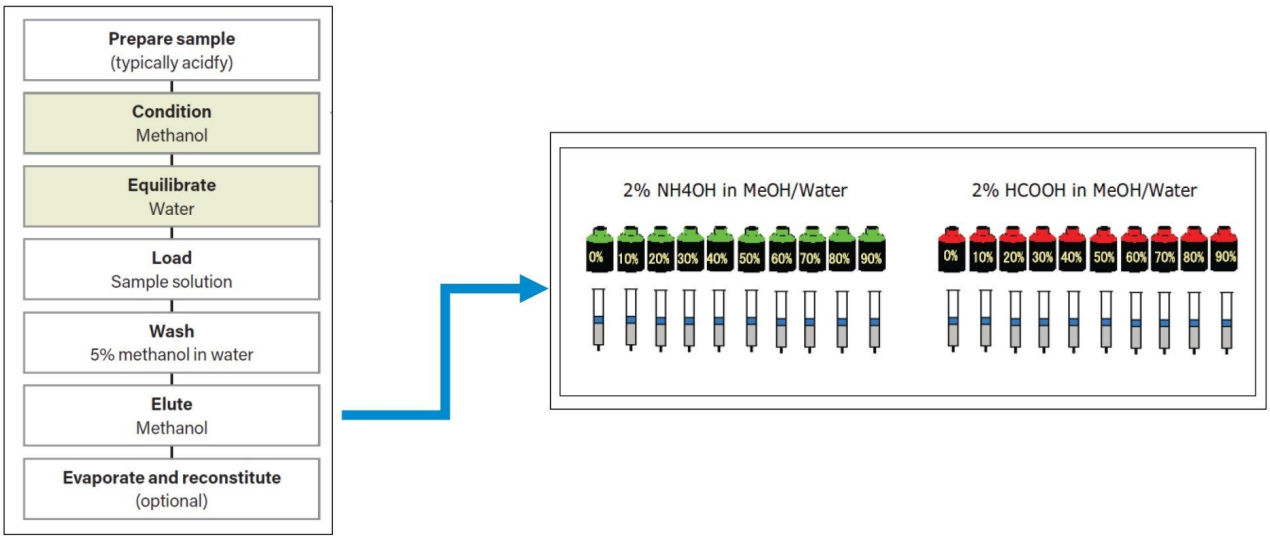


Figure 1. Visual representation of Oasis HLB 20 bottle method optimization approach from care and use manual.

Experimental

Chemicals, Reagents, Materials and Solvents:

A panel of 6 small molecule drugs with varied physio-chemical properties (Table 1) were used to showcase the 20 bottle method optimization approach for Oasis HLB SPE. All analytes were procured from Sigma Aldrich (St. Louis, MO, USA). LC-MS grade Methanol, water, acetonitrile, isopropanol, phosphoric acid, ammonium hydroxide, and formic acid were also purchased from Sigma Aldrich (St. Louis, MO, USA). K2 EDTA rat plasma was procured from BioIVT (Westbury, NY, USA). Oasis HLB µElution plates were procured from Waters Corporation (Milford, MA, USA).

Compound	LogP	Molecular weight
Palbociclib	1.8	447.5
Metoprolol	1.9	267.4
17-OH-Progesterone	3.2	330.5
Niflumic acid	3.7	282.2
Nortriptyline	4.5	263.4
Amitriptyline	5.0	277.4

Table 1. Analyte panel of small molecule drugs used.

Stock solutions, Calibration Curve, and QC Samples

A 1 mg/mL solution stock solution was prepared for each analyte by weighing accurately, approximately 1 mg of analyte and dissolving in appropriate volume of 100% Methanol. Working stocks of 100 µg/mL, 10 µg/mL and 1 µg/mL were prepared by diluting 1:10 in 95:5% (v/v) Water:Methanol. Calibration curve (1–1000 ng/mL) was prepared by spiking K2 EDTA rat plasma with 10 µg/mL stock solution using the Andrew+ pipetting robot at yield spiked matrix concentrations of 1000 ng/mL, 500 ng/mL and 250 ng/mL. These upper level calibration curve points were serially diluted (1:10) using Andrew+ pipetting robot and the Simple Serial Dilution preparation OneLab library protocol. For QC samples, 10 µg/mL stock solution was spiked into rat plasma to yield a HQC level at 750 ng/mL. The same Simple serial Dilution protocol was used to perform a 1:10 dilution to generate MQC and LQC levels at 75 ng/mL and 7.5 ng/mL respectively.

LC Conditions

LC system:	ACQUITY I-Class Plus UPLC with FTN
Column:	ACQUITY Premier HSS T3, 2.1 x 50 mm (p/n: 186003538)

Column temperature:	60 °C
Column temperature:	5 °C
Mobile phase A:	0.1% Formic Acid in Water
Mobile phase B:	0.1% Formic acid in Acetonitrile
Purge solvent:	25:25:25:25 Water:Acetonitrile:Methanol:Isopropanol
Injection volume:	2 µL

LC Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.500	95	5	6
0.50	0.500	95	5	6
4.00	0.500	5	95	6
4.40	0.500	5	95	6
4.50	0.500	95	5	6
5.00	0.500	95	5	6

MS Conditions

MS system:	Xevo TQ-XS
Ionization mode:	ESI+

Acquisition mode:	MRM
Capillary voltage:	3.1 kV
Desolvation temperature:	500 °C
Desolvation gas flow:	1000 L/Hr
Cone gas flow:	150 L/Hr
Collision gas flow:	0.15 L/Hr
Nebulizer:	7 Bar

Data Management

Instrument control software:	MassLynx™ (v4.2)
Quantification software:	TargetLynx™ (v4.2)

MRM Transitions

Compound	Precursor (*m/z*)	Product (*m/z*)	Cone voltage (V)	Collison energy (eV)
Prednisone	358.9	313.1	20	12
	358.9	147.1		28
Metoprolol	268.3	116.1	45	18
	268.3	98.1		18
17-OH progesterone	331.3	109.1	30	26
	331.3	97.1		22
Niflumic Acid	283.3	245.4	20	32
	283.3	145.7		38
Nortriptyline	264.6	104.9	40	20
	264.6	233.1		14
Amitriptyline	278.7	90.9	40	20
	278.7	104.9		20

Automation Platform

The Andrew Alliance Andrew+ Pipetting Robot, an automated liquid handling device, controlled by its cloud-based, OneLab Software was used design and execute the sample preparation and extraction protocols.

SPE Extraction

Reversed-phase (RP) SPE extraction was performed using an Oasis HLB 96-well micro elution plate (2 mg sorbent per well). The starting SPE protocol used for this work is shown in Figure 2. Briefly, 100 µL samples were pre-treated with 100 µL of 4% phosphoric acid. Oasis HLB µelution plate was conditioned with 200 µL Methanol and equilibrated with 200 µL of Water. The acid pre-treated samples were then loaded onto the SPE plate. The plate was washed with 200 µL of 5% methanol in water. Analyte elution was performed in 2 steps of 50 µL each with the 20 solutions as described in Figure 2. The SPE sample eluent was then diluted with 100 µL water and injected onto the LC-MS/MS system for analysis. The deck layout for the Andrew+ Pipetting Robot with Extraction+ and an example of a visual representation of the OneLab protocol used for this experiment are shown in Figure 3 and Figure 4 respectively.

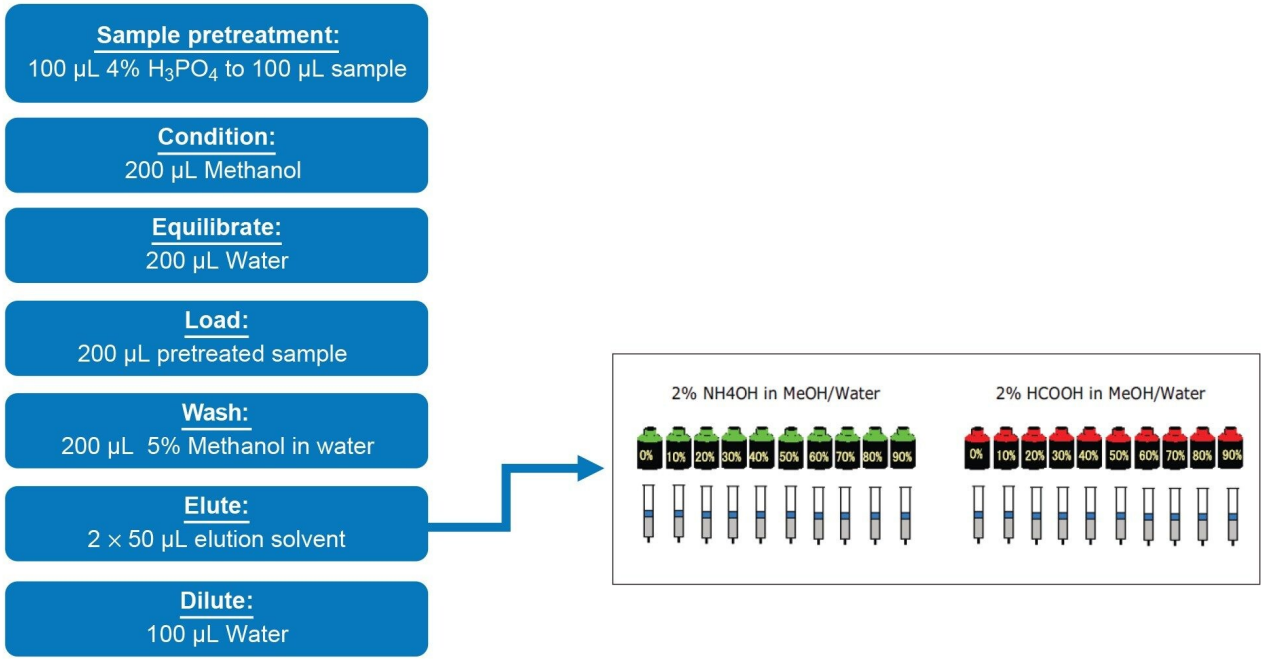
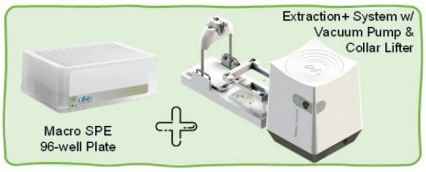


Figure 2. Oasis HLB SPE protocol used for method development.



Position	Domino
1–4	Tip insertion system
5	Extraction+
6	Storage plate
7	Microtube
8	50 µL conical centrifuge tube
9–10	8-channel pipette reservoir

Figure 3. Deck layout for Andrew+ pipetting robot with Extraction+.

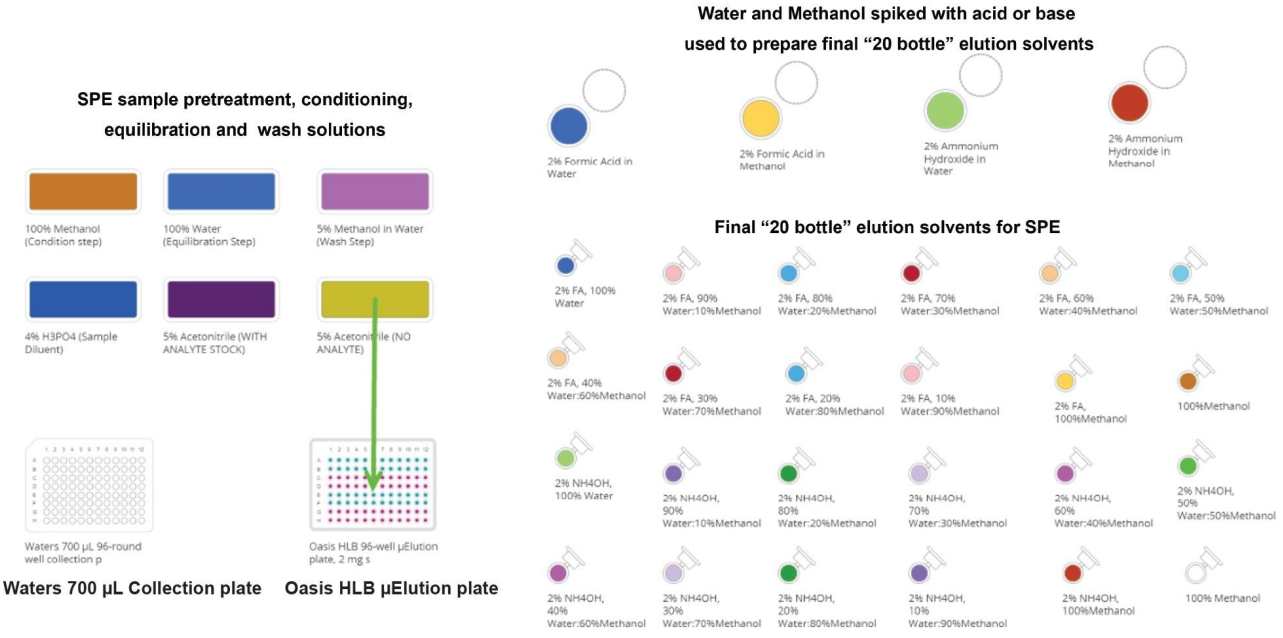


Figure 4. Representative visual illustration of OneLab protocol steps.

Results and Discussion

Robust, sensitive and reproducible assays are the lifeline of bioanalytical laboratories. Developing and validating these assays in a time and cost-efficient manner is critical to ensuring optimal use of personnel and resources. As such, scientists in these laboratories strive to get to the final method in the simplest way possible. For SPE, this usually means starting with the generic protocol provided in the care and use manual for Oasis HLB and performing an experiment in neat solution to understand the % recoveries and levels of sensitivity that can be achieved without any sample preparation method optimization. For many analytes, the starting Oasis HLB starting protocol provides sufficient % recovery and sensitivity for the assay to be fit-for-purpose. In instances where the desired levels of sensitivity cannot be reached, the next step is to optimize the starting protocol using the 20-bottle approach as described in the Oasis HLB care and use manual.

The 20 bottle SPE method optimization approach describes an elegant experimental design which varies the pH and the organic content of the elution solution to determine which condition provides the best probability of eluting the compound of interest from the sorbent bed. The elution solvent composition varies from 0–100% organic (generally methanol) with either 2% ammonium hydroxide or 2% formic acid. The acidic or basic nature of the elution solvent helps alter the pH, whereas the organic content helps dissociate analytes from the sorbent bed and improve solubility. Additionally, because this experimental approach provides an elution profile for the analytes at different concentrations of organic solvent, the same data can also be used to determine the highest % organic that can be used as a wash solvent which will eliminate unwanted matrix components without losing any analyte from the SPE sorbent, further enhancing method selectivity. The final experiment for SPE method development usually involves testing the optimized wash and elution solvents to extract analytes spiked in the matrix of interest to determine recovery and matrix effects.

Here, we have used a panel of 6 analytes to highlight the 20-bottle method optimization approach. The aim of this piece of work was to determine best wash and elution solvent compositions that facilitate high recovery of all analytes in the panel. To simplify the method optimization for Oasis HLB even further, we created a method in OneLab software and used Andrew+ pipetting robot with Extraction+ to automate the full experiment, from creating the 20 elution solvents to be used in the method, as well as performing SPE using the Oasis HLB µElution plate.

For each analyte in the panel, the % recoveries (y-axis) observed at each of the elution solvent conditions (x-axis) were plotted to provide an elution profile. A representative elution profile for Nortriptyline is shown in Figure 5. The orange trace represents elution profile using acidified (2% formic acid) elution solvents and the blue trace

represents elution profile using basic (2% ammonium hydroxide) elution solvents. For Nortriptyline, we observe recoveries >80% starting at 70% acidified methanol and 90% basic methanol. The best elution condition for this analyte will be at these organic solvent concentrations. Nortriptyline does not elute from the SPE sorbent upto 50% acidified Methanol and 60% basic methanol. The ideal wash solvent will be below this organic solvent concentration.

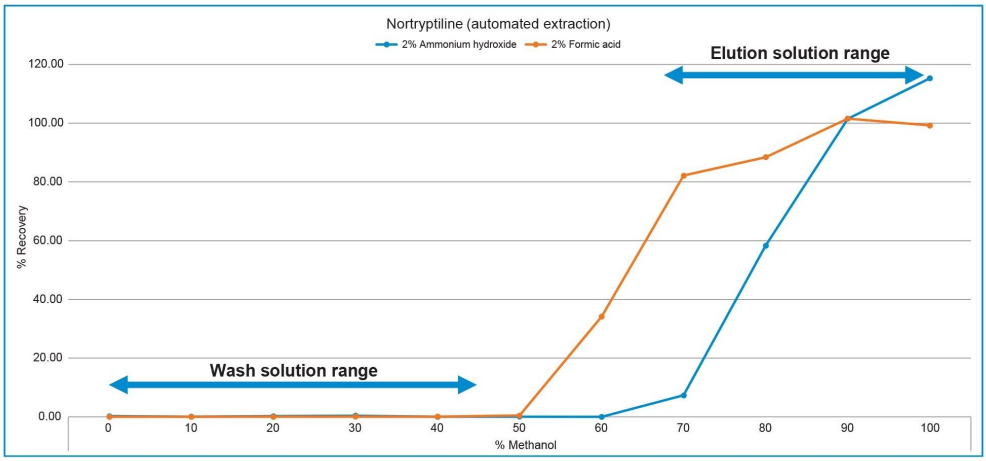


Figure 5. Representative elution profile for Nortryptiline.

Table 2a and 2b show heat maps for the observed recoveries for all analytes in the panel using basic and acidic elution solvents respectively. For each analyte within acidic or basic elution solvent conditions, the % recoveries observed increase from red to green boxes. Picking the ideal elution solvent is a balance between the % organic concentration that provides high recovery for analyte/s of interest without eluting potentially undesirable components from the SPE sorbent. Acceptable recoveries for most analytes in the panel were observed at 2% formic acid in 80% methanol. This was chosen as the final elution solution for the panel. Similarly, the ideal wash solution is the highest organic concentration is one which allows removal of most unwanted matrix components without eluting any analyte of interest. Based on the data observed, 30% methanol was chosen as the wash solvent for this panel of analytes.

(a) 2% Ammonium hydroxide						
% Methanol elution	Amitriptyline	Niflumic acid	17-OH-Progesterone	Prednisone	Nortriptyline	Metoprolol
0%	0.22	0.01	0.65	0.29	0.06	0.06
10%	0.06	0.02	0.06	0.05	0.09	0.09
20%	0.22	0.02	0.18	0.16	0.15	0.07
30%	0.38	0.07	0.07	0.28	0.30	0.01
40%	0.05	0.98	0.10	0.80	0.12	0.56
50%	0.08	31.46	0.43	16.09	0.01	5.03
60%	0.01	82.87	4.15	57.79	0.16	37.14
70%	7.32	85.74	26.04	87.16	9.30	79.98
80%	58.32	93.17	68.48	88.64	41.81	88.69
90%	101.51	98.07	81.45	100.49	92.62	97.58
100%	115.29	94.69	93.22	95.62	98.12	90.12

(b) 2% Formic acid						
% Methanol elution	Amitriptyline	Niflumic acid	17-OH-Progesterone	Prednisone	Nortriptyline	Metoprolol
0%	0.02	0.00	0.07	0.03	0.05	0.21
10%	0.04	0.00	0.08	0.08	0.06	0.31
20%	0.03	0.00	0.02	0.11	0.06	0.40
30%	0.06	0.00	0.05	0.11	0.06	5.73
40%	0.05	0.00	0.01	0.11	0.06	29.62
50%	0.44	0.01	0.04	0.17	0.28	39.17
60%	34.15	0.02	0.05	11.25	19.34	56.49
70%	82.15	0.10	2.37	57.88	57.81	58.52
80%	88.43	76.53	73.17	76.25	107.44	68.13
90%	101.54	47.70	73.62	73.40	87.56	95.84
100%	99.22	51.24	78.34	86.38	92.88	83.22

Table 2. Heat map of the elution profile for all analytes in the panel.

In a subsequent experiment, the wash and elution conditions from the 1st experiment were used to perform SPE for the analyte panel spiked in rat plasma at 1 µg/mL. The recoveries using the final wash/elution solvent conditions between analytes spiked in neat solution (5% methanol) and rat plasma were compared. Figure 6 shows comparable recoveries of >80% for all analytes using the final optimized wash and elution conditions. Matrix effects were also calculated for each analyte in the panel. As shown in figure 7, the matrix effects for all analytes except Amitriptyline were below 15% using the final SPE conditions. Although not required, matrix effects for Amitriptyline can be further optimized by choosing different wash/elution conditions, evaluating different MRM transition, or acidic/basic pre-treatments before SPE.

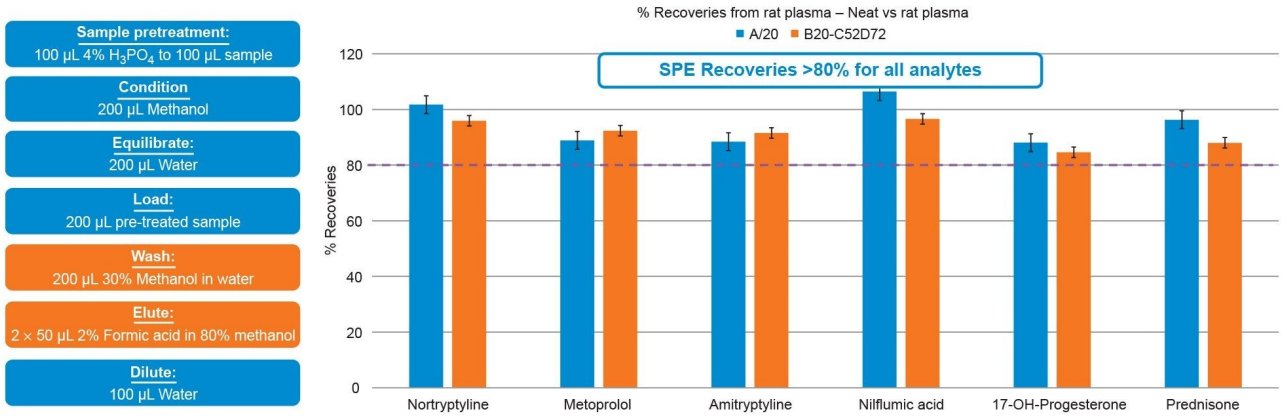


Figure 6. Final SPE protocol based on optimization results.

Comparison of % recoveries for analyte panel spiked in neat (5% Methanol) and rat plasma using final optimized SPE protocol.

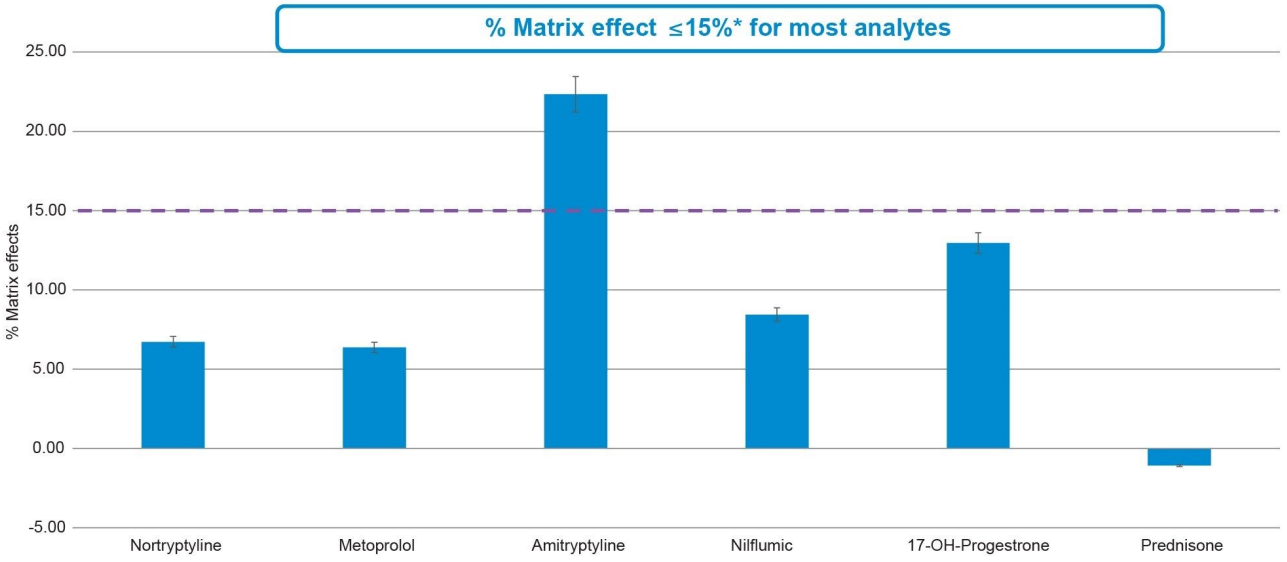


Figure 7. % Matrix effects for analyte panel spiked in rat plasma and extracted using final SPE protocol.

The final optimized SPE protocol using 30% methanol for the wash step and 2% formic acid in 80% methanol as the elution step was applied to a sample set representing a typical bioanalytical batch. Nortriptyline was used as

a model analyte for this assay.

Nortriptyline was spiked in K2 EDTA rat plasma using a 'Simple Serial Dilution' method from OneLab to be used with Andrew+ pipetting robot. Duplicates for the calibration curve (1–1000 ng/mL) and QC points (7.5 ng/mL, 75 ng/mL, and 750 ng/mL) were created using a 10 µg/mL stock solution. 'Bioanalytical SPE method' was downloaded from the OneLab library and used to extract Nortriptyline from rat plasma. The final SPE protocol used for this extraction is shown in Figure 6. The extracted samples were analysed using the LC-MS methods described in the experimental section.

Calibration curve and QC statistics (N=2) observed for Nortriptyline are described in Table 3. Nortriptyline was linear from 1–1000 ng/mL with a linear 1/x weighing and r2 of 0.9943. The mean % accuracy for the calibration curve for Nortriptyline was 104.8%, and mean deviation was -8.73%. All QC levels for Nortriptyline passed the bioanalytical method validation criteria of % accuracy and % deviation of +/- 15%. The mean accuracies for QC levels ranged from 96–110% and mean % deviation was within -11.9–5.4%. Representative QC chromatograms for Nortriptyline showed a linear increase in MS area counts with increase in analyte concentration (Figure 8).

Nortriptyline			
Calibration curve range	**Linear fit**	**Mean % accuracy (N=2)**	**Mean % deviation (N=2)**
1–1000 ng/mL	1/x	104.8	-8.7

QC level	Expected concentration (ng/mL)	Observed concentration (ng/mL)	Mean % accuracy (N=2)	Mean % deviation (N=2)
LQC	7.5	7.2	96	-11.9
MQC	75	81	108	5.4
HQC	750	825	110	4.1

Table 3. Calibration curve statistics (3a) and QC statistics (3b) for Nortryptiline extracted from rat plasma using final optimized SPE protocol.

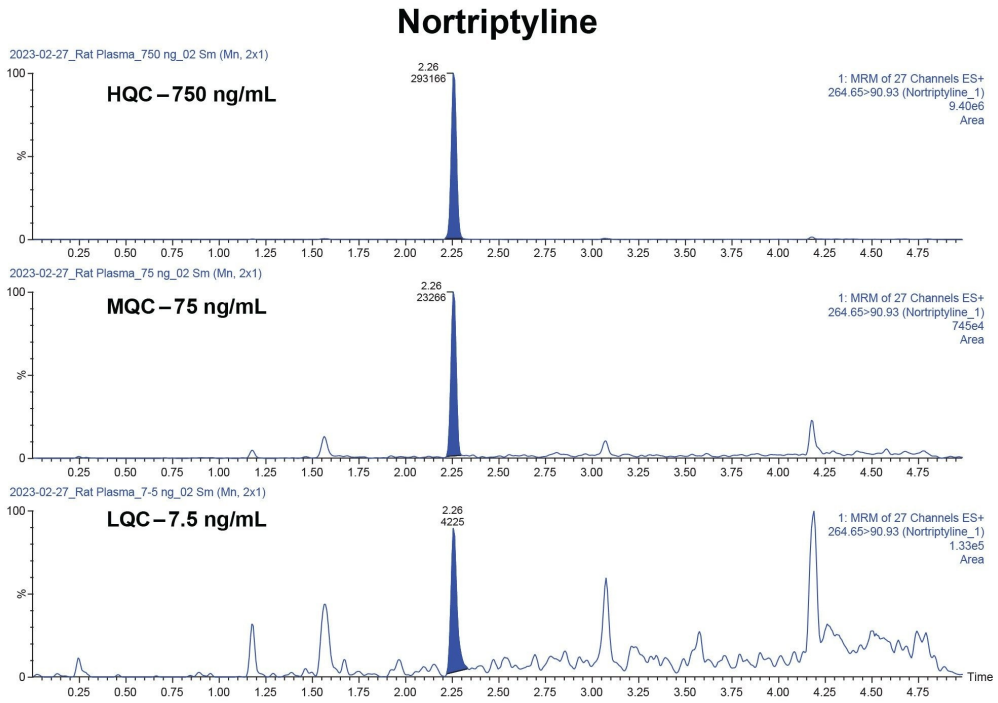


Figure 8. Representative QC chromatograms for Nortryptiline show increase in MS area counts with increase in analyte concentration.

Conclusion

We applied the 20-bottle method optimization for our panel of 6 analytes using the Andrew+ pipetting robot and Extraction+ connected device. Based on the results, the best wash and elution solvents for our panel were 30% Methanol and 2% formic acid in 80% Methanol respectively. Using these optimized conditions, we performed a representative bioanalytical run to quantify Nortriptyline from rat plasma. Nortriptyline extracted from rat plasma was linear from 1–1000 ng/mL. All calibration curve and QC points met the bioanalytical method validation guidelines of +/- 15%.

Solid Phase Extraction (SPE) is the method of choice in bioanalytical/DMPK labs when high selectivity, high recoveries and low matrix effects are desired. However, the method development for SPE is perceived to be challenging, with the need to optimize multiple steps. Here, we have highlighted an easy, elegant approach to

method optimization for revered-phase Oasis HLB µelution using the 20-bottle approach. This single experiment optimizes for wash and elution solutions. Creating a one-click, easy to download and use OneLab method four use with Andrew+ pipetting robot and Extraction+ connected device makes method development even more streamlined and easy, thereby saving time and ensuring consistency across all user experience levels.

References

1. Oasis HLB Cartridges and 96-Well Plates, Waters Care and Use Manual, 715000109 < https://www.waters.com/webassets/cms/support/docs/715000109.pdf> .

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ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

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