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Note d'application

Easy and Robust Automated Sample Preparation and Extraction for LC-MS/MS Bioanalytical Workflows

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Abstract

The following work demonstrates the capabilities of the Andrew+™ Pipetting Robot in combination with the Extraction+ Connected Device for fully automated bioanalytical sample preparation for a variety of extraction techniques including protein precipitation (PPT), supported liquid extraction (SLE), reversed-phase solid phase extraction (SPE) and mixed-mode SPE. Most of the protocols were adapted from existing OneLab™ Library methods, minimizing the method development time. Flow-through waste collection of Extraction+ enabled fully automated "walk-away" performance. Results from all automated bioanalytical protocols demonstrated excellent accuracy and precision for all techniques, easily meeting bioanalytical regulatory guidelines. This automation platform enables easy implementation, excellent accuracy and precision and the flexibility to execute a variety of quantitative bioanalytical techniques.

Benefits

- Easy-to-use OneLab Software with data visualization for creating and transferring methods
- Fully programmable vacuum pressure profiles with Extraction+ Connected Device reduce extraction performance variability
- Automated liquid handling and sample preparation increases efficiency, allowing the user to perform other

tasks

- Full “walk-away” automation with no or minimal user intervention steps, mitigates the risk of manual error with liquid handling capabilities
- Ready-made, downloadable protocols from OneLab minimize protocol development time

Introduction

Bioanalytical sample preparation methods can range from simple techniques such as dilute and shoot or protein precipitation to more targeted and specialized methods such as liquid-liquid extraction, solid phase extraction (SPE) or immunoaffinity purification (Figure 1). Generally, the simpler techniques have wider applicability and require minimal method development with a trade-off of limited cleanliness and sensitivity. The more specific techniques offer superior cleanliness, specificity and sensitivity but have more limited applicability and may require more method optimization.

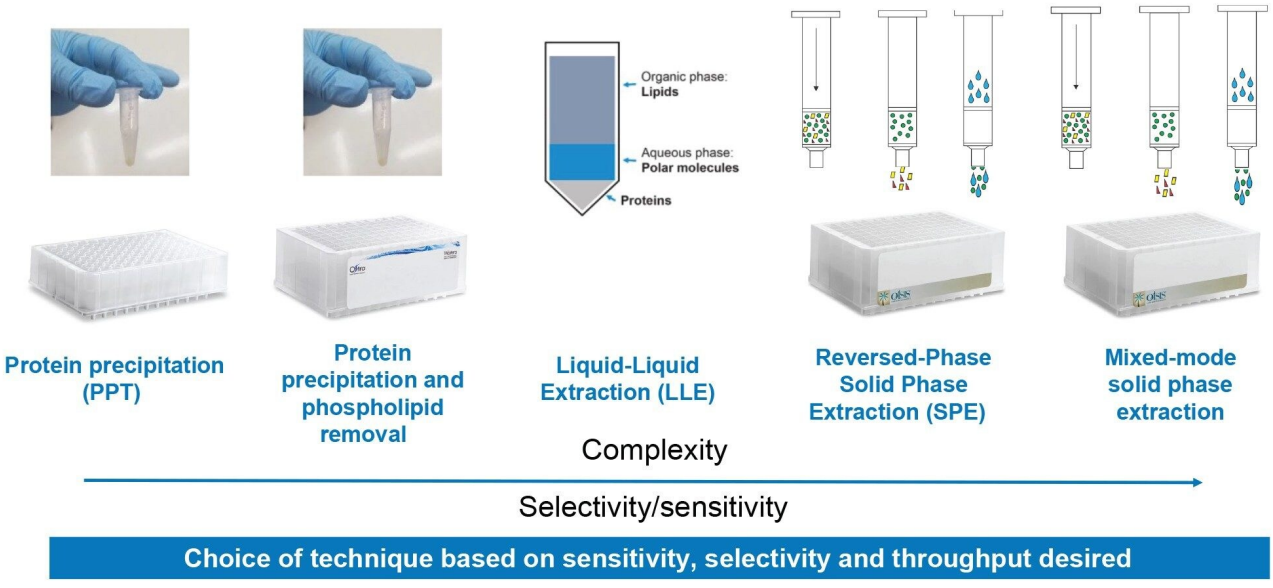


Figure 1. Graphical illustration of common bioanalytical extraction techniques.

Regardless of the sample preparation technique used, one of the challenges of bioanalytical sample preparation in modern laboratory settings is achieving consistent results both within and between batches. Technique dependent variables such as pipetting consistency and other user dependent variables can impact accuracy and repeatability, especially in laboratory settings with substantial personnel turnover. Other sources of error include pipetting incorrect reagents, accidentally skipping steps in the procedure and errors in sample tracking or transposition. Automating the sample preparation workflow can minimize or eliminate many of these sources of variability. It also has the added advantage of freeing up scientists for other tasks and reducing the risk or repetitive-stress injuries from pipetting.

In this work, the Andrew+ Pipetting Robot configured with the Extraction+ Connected Device was used to fully automate the sample preparation and extraction of the therapeutic drug, apixaban from plasma. Several common bioanalytical techniques were employed including protein precipitation (PPT), PPT with phospholipid (PL) removal, supported liquid extraction (SLE), reversed-phase (RP) SPE, RP-SPE with PL removal and mixed-mode SPE. Each method including all pipetting steps, vacuum settings used in extraction, waste disposal and final collection was fully automated. Qualitative assessments, including analyte recovery, matrix effects and residual PLs were used to compare efficiency and cleanliness of the methods as well as to screen appropriate mixed-mode SPE sorbents. This was followed by evaluation of quantitative performance including linearity, accuracy and precision. For all extraction techniques, calibration standards and quality control (QC) accuracies with respective % RSDs, used as a measure of robustness, were <10%, with many <5%. These quantitative results demonstrate that the Andrew+ Pipetting Robot configured with the Extraction+ Connected Device has the flexibility and required repeatability for the most common bioanalytical sample extraction techniques. This can benefit labs by improving method consistency while minimizing or eliminating many of the errors and variability associated with manual sample preparation. The Andrew+ Pipetting Robot with the Extraction+ Connected Device enabled fully automated “walk-away” performance with excellent results for this wide variety of techniques, eliminating the risk of user error, freeing up scientists for other tasks and providing consistent performance independent of user technique or experience.

Experimental

Chemical and Solutions

Apixaban was purchased from Cerilliant (www.cerilliant.com). 13C-d3 Apixaban was obtained from Cayman

Chemicals and used as an internal standard (IS). Stock solutions (1 mg/mL) were prepared in methanol. Rat plasma (K_3EDTA) was purchased from Innovative Research (www.innov-research.com). Daily working solutions for curve and QC generation were prepared in plasma. LC-MS grade formic acid and phosphoric acid were purchased from Sigma Aldrich. Tert-butyl methyl ether MTBE was obtained from Avantor sciences.

Sample Preparation Extraction Devices

Sirocco Protein Precipitation plates Ostro Protein Precipitation & Phospholipid Removal Plates, Oasis HLB, Oasis PRiME HLB, Oasis sorbent selection plates and Oasis MCX plates were all obtained from Waters. Supported Liquid Extraction (SLE) plates (p/n: 96260–1) were obtained from Analytical Sales and Services (analytical-sales.com).

Standard Curve and Quality Control Sample Preparation

Working stock solutions of apixaban and its internal standard apixaban 13C-d3 were prepared in methanol and 10 µg/mL and 100 µg/mL, respectively. For recovery and matrix effects experiments, pre-spiked plasma solutions containing 100 ng/mL apixaban were prepared from the stock solution. Working calibrators and QC samples in plasma were prepared from the working stock solution at concentrations ranging from 2–500 ng/mL. Working IS solutions were prepared from the 100 µg/mL working stock solution as required for each sample preparation workflow.

Automation Platform

The Andrew+ Pipetting Robot, equipped with the Extraction+ Connected Device and controlled with the cloud-based OneLab software, was used to design and execute the sample preparation and SPE extraction protocols.

Sample Extraction protocols

The OneLab Library protocols used for each technique are listed in Table 1 and graphical diagrams of the protocols used for each sample preparation method are shown in Figure 2. In each case, the manufacturers' instructions for appropriate volumes and solvents were followed. All steps were fully automated by the Andrew+ system except for the vortexing step for the PPT protocol and the evaporation step for the SLE protocol.

Standard OneLab protocols from the OneLab Library https://onelab.andrewalliance.com/app/lab/D8xeYomN/library < https://onelab.andrewalliance.com/app/lab/D8xeYomN/library> , were downloaded and used for Ostro, Oasis HLB, Oasis HLB PRiME, and the Mixed-mode screening protocol. The 2 x 4 method development protocol also

included steps for spiking the extracted samples to assess analyte recovery. New protocols were created for the PPT preparation using the Sirocco plate and the SLE plate.

OneLab Protocols

Sample prep procedure	Starting library protocol
PPT with Sirocco	**New protocol**
PPT with Ostro	Ostro protein precipitation
SLE	**New protocol**
RP-SPE (Oasis HLB)	Automated bioanalytical SPE
RP-SPE with PL removal (Oasis HLB PRiME)	Automated bioanalytical SPE
Mixed-mode screening	Oasis 2 × 4 method development
Oasis MCX 96-well plate	Automated bioanalytical SPE

Table 1. Table of sample preparation methods, starting OneLab library protocols and the links to the protocols in the OneLab library.

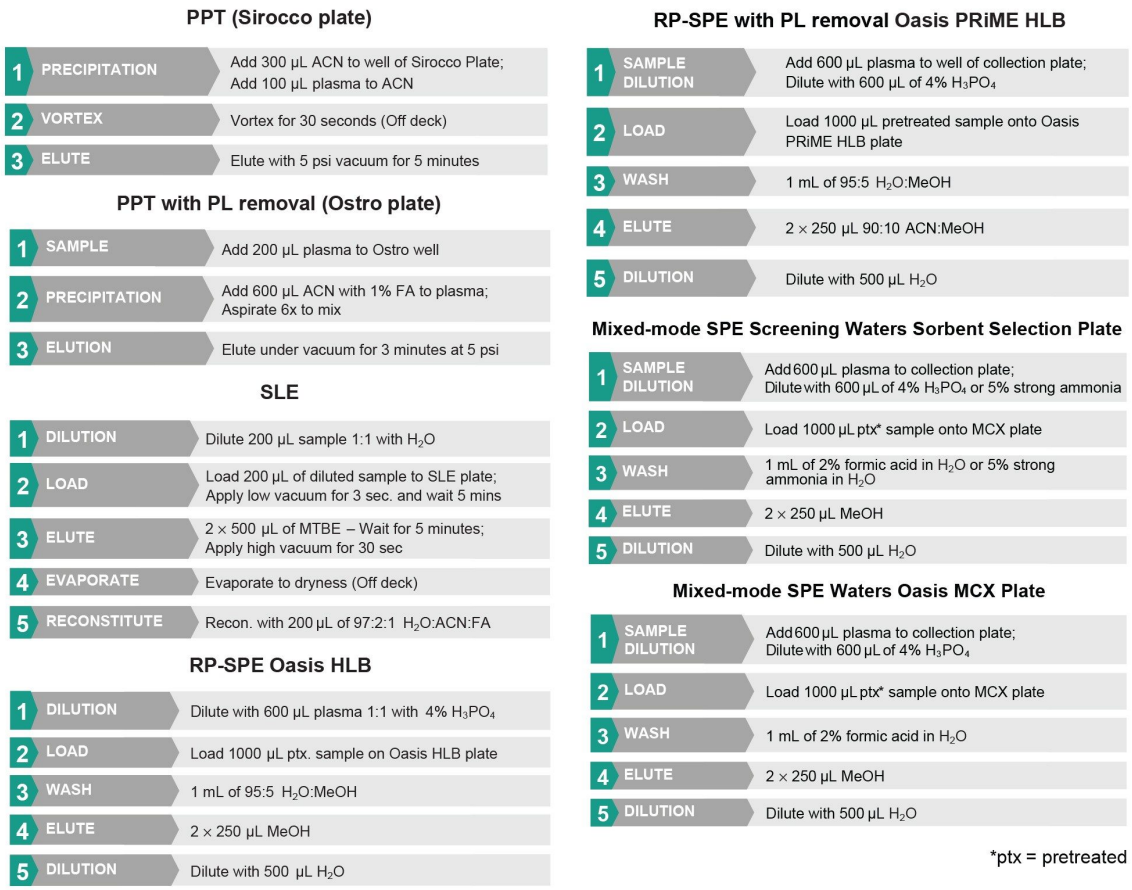


Figure 2. Graphical representations of all sample preparation methods used in this work. All methods were based on manufacturers' guidance for volumes and solvents. ptx-pretreatment.

LC Conditions

LC system:	ACQUITY I-Class UPLC (FL)
Mobile phase A:	0.1% Formic Acid 100%in MilliQ water
Mobile phase B:	0.1% Formic Acid in 100% Acetonitrile
Weak wash solvent:	Water:methanol (90:10 v/v)

Strong wash solvent:	Acetonitrile: Isopropanol: Water: Methanol (25:25:25:25 v/v/v/v)
Detection:	Xevo TQ-S Mass Spectrometer
Column(s):	ACQUITY UPLC BEH C18 Column, 1.7 µm, 2.1 mm x 50 mm (p/n: 186002350)
Column temperature:	35 °C
Sample temperature:	10 °C
Injection volume:	5 µL
Flow rate:	0.5 mL/min

LC Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.5	95	5	6
4.0	0.5	0	100	6
4.5	0.5	0	100	6
4.6	0.5	95	5	6
5.0	0.5	95	5	6

MS Conditions

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

Acquisition range:	MRM
Capillary voltage:	2.0 kV
Cone voltage:	30 V
Desolvation temperature:	500 °C
Desolvation flow:	1100 L/Hr
Cone gas flow:	150 L/Hr
Collision gas flow:	0.2 mL/min
Nebulizer gas flow:	7 Bar

Data Management

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

Compound	Ion Mode	M+H+	Fragment ion (Primary)	CE1	Fragment ion (Confirmatory)	CE2
Apixaban	ESI-Pos	460.2	443.2	30	199.1	35
Apixaban-C13-D3 (IS)	ESI-Pos	464.4	447.2	30	203.1	35

Table 2. Apixaban and its internal standard, Apixaban-C13-D3 used for Andrew+ Pipetting Robot configured with the Extraction+ SPE performance evaluation with their respective MRM precursor and fragments used for MS analysis.

Results and Discussion

Automation

The Andrew+ Pipetting Robot was used with the Extraction+ Connected Device to extract a target pharmaceutical (Apixaban) from plasma samples using a variety of common sample preparation techniques described above. All pipetting, reagent additions, sample mixing, sample pretreatment and extraction device manipulations were fully automated. The Extraction+ Connected Device enabled flow-through waste collection, eliminating the need for manual disposal of liquid waste. Placement of collection labware in the Extraction+ manifold and subsequent removal to the microplate dominos was also automated. Figure 3 shows the Andrew+ pipetting robot configured with the Extraction+ Connected Device and Figure 4 shows the Extraction+ Connected Device and its accessories.

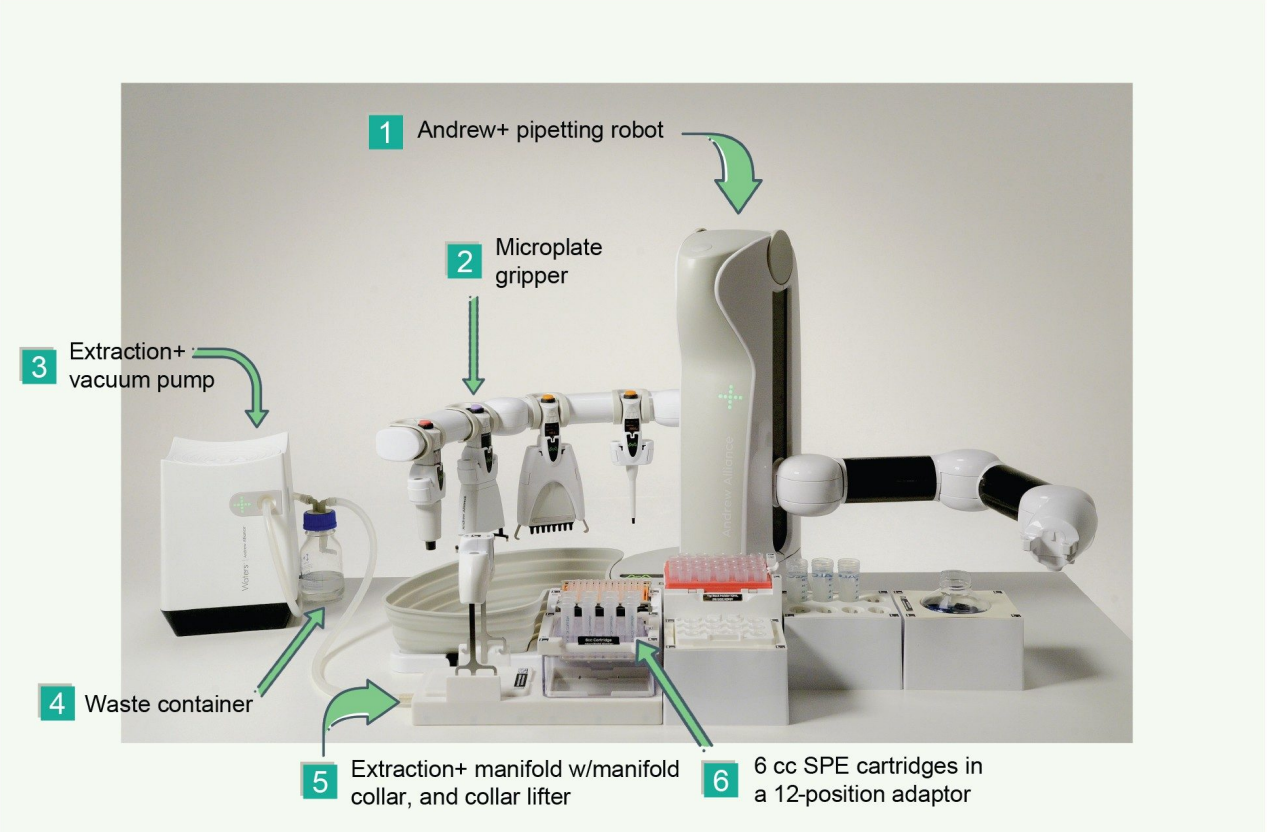


Figure 3. Andrew+ Pipetting Robot configured with the required Dominos, the Andrew Alliance Bluetooth electronic pipettes and microplate gripper on the tool stand and the Extraction+ Connected Device including the Extraction+ connected vacuum pump, flow-through waste container, Extraction+ manifold with the manifold collar, the integrated collar lifter, and SPE cartridges in the corresponding adaptor.

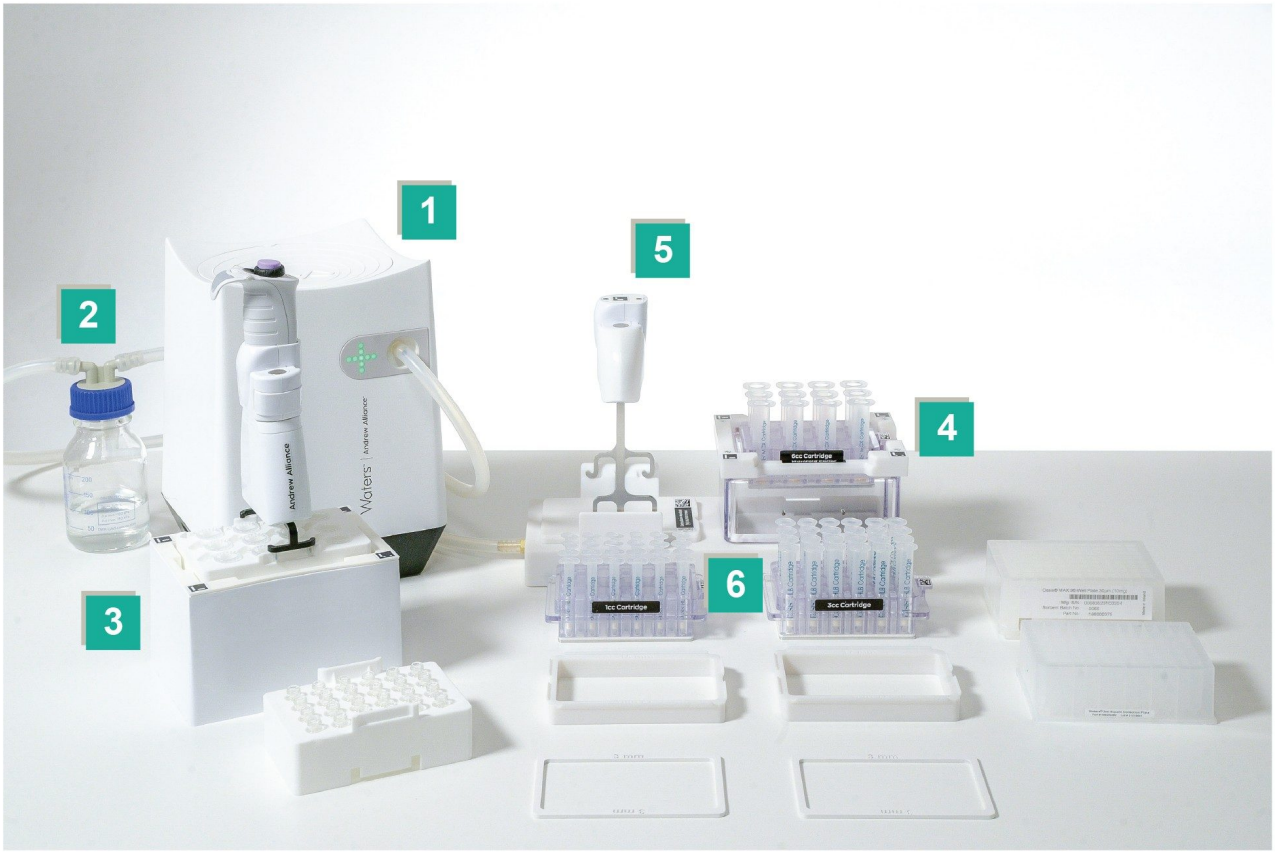


Figure 4. The Extraction+ Connected Device showing the integrated vacuum pump (1), the flow-through waste collection bottle (2), Domino with sample collection racks (3), the extraction manifold (4) with the collar lifter (5) to move the manifold, and racked SPE cartridges (6). Shims for adjusting collection plate height are also shown in the foreground.

One of the features of the OneLab software is the generation of equipment lists, protocol visualizations and deck layouts for all protocols. Figures 5–7 show examples of these for the extraction of apixaban from plasma using Oasis MCX 96-well plates. Similar layouts and visualizations were created for all 7 protocols executed during this work. This ensures that all necessary equipment and consumables are available and on-deck prior to starting any protocol. It also guides the user on setting up all the required dominos and connected devices in the proper position and contributes to the ease of use of the system.

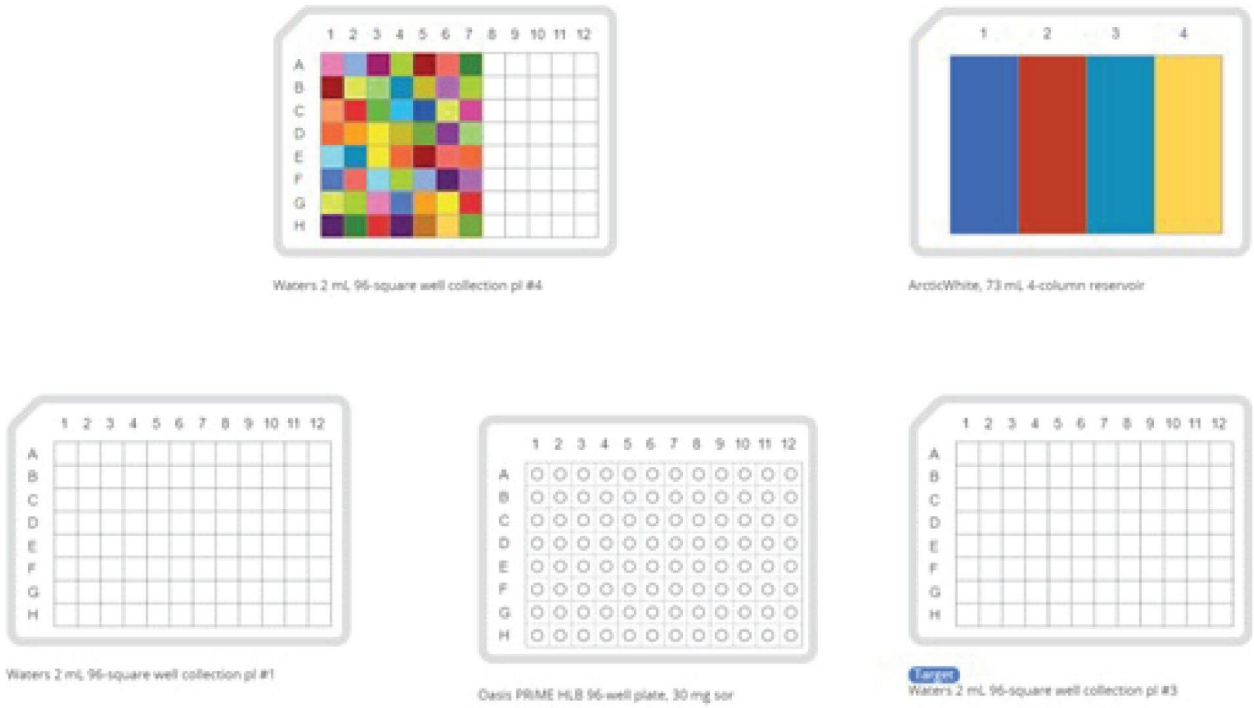


Figure 5. Extraction of Apixaban using Oasis MCX 96-well plate. The OneLab software's protocol visualization of an SPE method is shown. Individual samples are color coded in the 96-well sample plate in the upper left and the final target plate is designated at the lower right of the figure.

Apixaban extraction using Oasis MCX 96-well plate

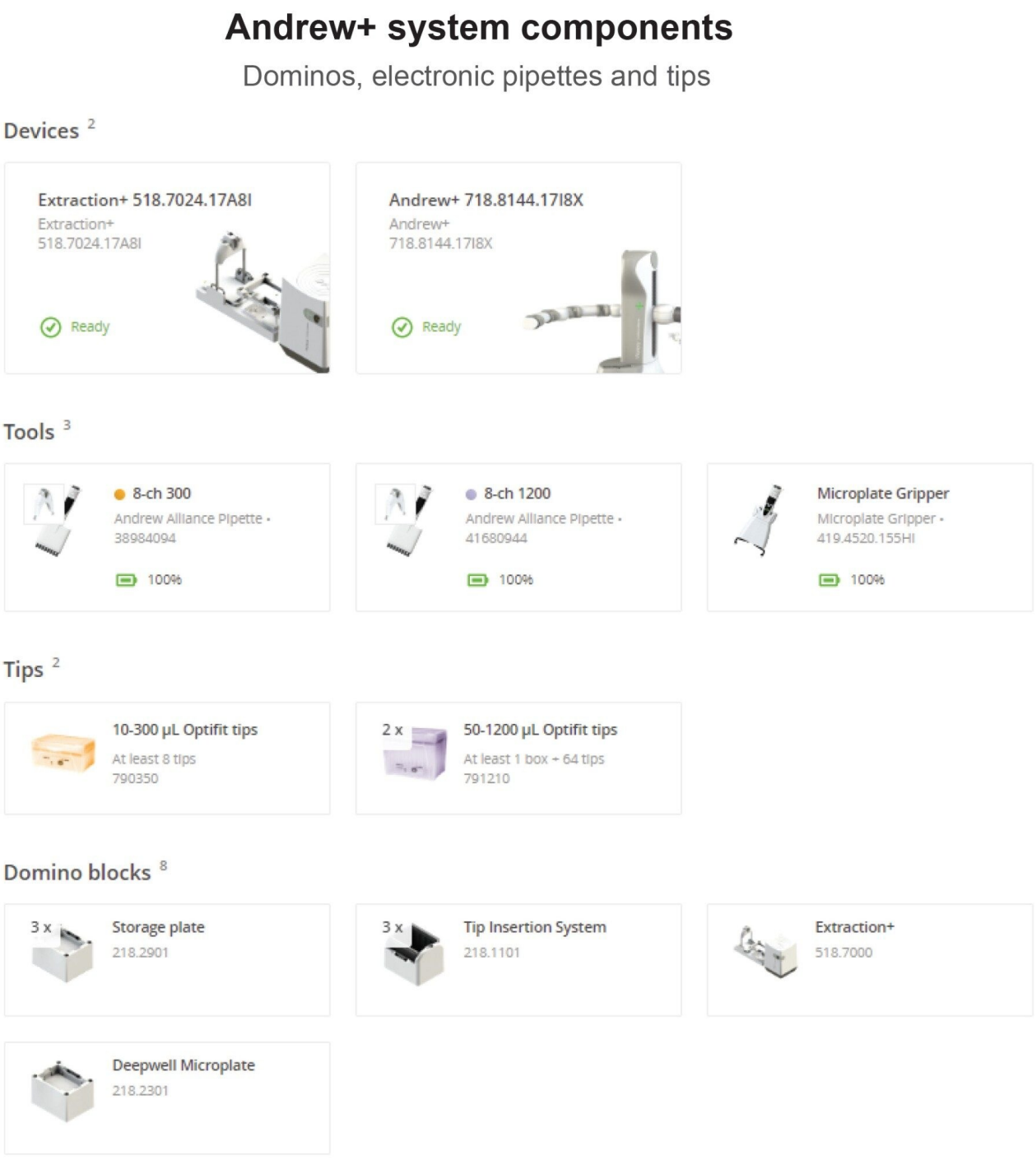
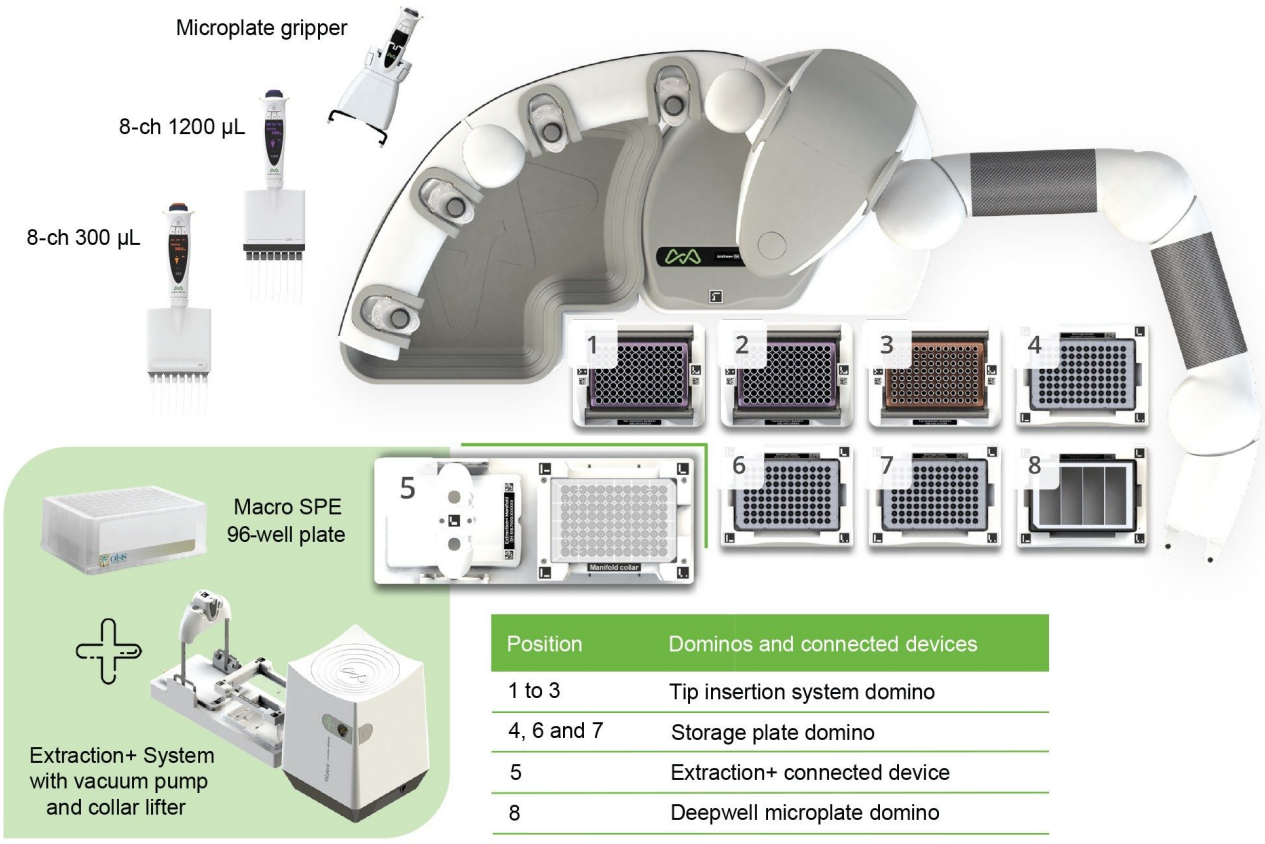


Figure 6. Extraction of Apixaban using Oasis MCX 96-well plate. The OneLab protocol's equipment list and protocol visualization are shown above. All required dominos, connected devices, tools, pipette tips are shown. OneLab generated similar lists for all other protocols as well.



Position	Dominos and connected devices
1 to 3	Tip insertion system domino
4, 6 and 7	Storage plate domino
5	Extraction+ connected device
8	Deepwell microplate domino

Figure 7. Extraction of Apixaban using Oasis MCX 96-well plate. The OneLab protocol's deck layout for the mixed-mode extraction of Apixaban is shown above, illustrating the placement of all items. Dominos and required devices are listed below the figure. Similar layouts were generated for all protocols used in this current work.

Recovery and Matrix Effects

A key step in any bioanalytical procedure is the evaluation of extraction efficiency and cleanliness. This is done by calculating the recoveries and matrix effects for the target analytes. Figure 8 shows the recovery and matrix

effects results from the sample preparation procedures. The sample preparation techniques are ordered by increasing selectivity, starting with the more universal methods such as protein precipitation and progressing to the more selective and specific mixed-mode SPE procedures. A general trend of improved recovery and decreased matrix effects were seen with the more specific methods. Both PPT techniques (Sirocco and Ostro) had acceptable recoveries but substantial matrix effects. SSLE prepared samples had the least recovery of all techniques. It should be noted that minimal optimization was performed for this or any technique. It is possible that this performance would improve with some optimization experiments. Looking at the SPE techniques, this pattern of improved performance is more evident. All SPE techniques had good recoveries (>80%), but the magnitude of the matrix effects decreased from -40% for Oasis HLB to -13.6% for HLB PRiME with negligible matrix effects for Oasis MCX.

In addition to executing routine protocols, Andrew+ can also be used for method development and optimization. In this case, the use of the 2 x 4 protocol from the OneLab library enabled the screening of all four mixed-mode sorbents. The other mixed-mode solvents, WCX and MAX had negligible recovery and are not shown in the figure. It should be noted that the eluate from the first elution was used for the mixed mode sorbents. Apixaban is not ionizable and is not expected to bind to the mixed mode sorbents via ion exchange. Thus, the ion-exchange character of the mixed-mode sorbent is used to provide additional cleanup vs HLB or HLB PRiME. Since Oasis MCX gave superior performance vs. WAX in the form of lower matrix effects, that sorbent was used for subsequent quantitative work. The use of the Andrew+ Pipetting Robot with the Extraction+ Connected Device allowed the rapid screening of all sample preparation techniques. The availability of pre-configured protocols for many of the techniques also helps to save time and quickly evaluate the methods.

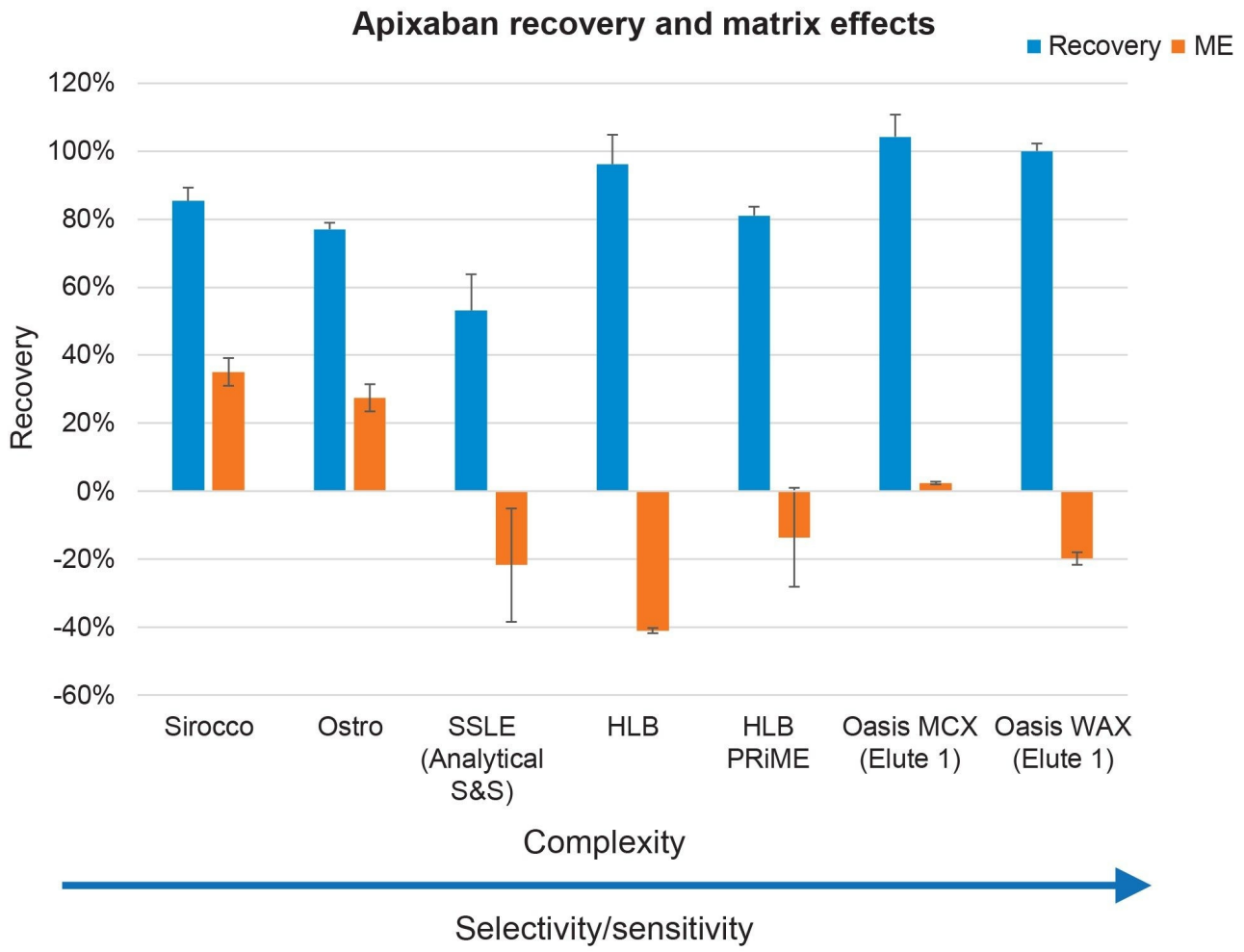


Figure 8. Recovery and matrix effects resulting from the extraction of Apixaban from plasma using the techniques listed above. All pipetting, vacuum steps, waste handling, and dilutions were performed by the Andrew+ Pipetting Robot configured with the Extraction+ Connected Device.

Quantitative Results

Tables 3 and 4 summarize the quantitative results from the Andrew+ and Extraction+ extraction of standards and QC samples using the sample preparation techniques described above. One of the key advantages of automating sample preparation is the consistency and reproducibility that can be achieved. Calibration curves ranged from 2–500 ng/mL for all sample preparation techniques. Table 3 shows that calibrator accuracy ranged from 86–111% with %RSDs <15% (*N*=3), with many in the single digits. This easily meets FDA guidance for

bioanalytical method validation. A summary of quality control results associated with each technique is shown in Table 4. Although there were significant differences in recovery and matrix effects from the different techniques (Figure 8), quantitative performance was excellent for all extraction techniques. As shown in Table 4, mean accuracies were all within 10% of nominal values. Precision was excellent as well. All %RSD values were in the single digits, and all but one were under 5%, again easily meeting FDA guidelines for method validation.

In addition to the excellent reproducibility demonstrated, one of the key advantages of automation is the minimization or elimination of operator errors that can occur during manual sample preparation. These can include things such as pipetting the wrong samples, either via transposition or possibly missing a row or column of samples. Errors in reagent addition are also eliminated. The OneLab software ensures that the correct reagent and the correct amount of each reagent is added at the appropriate time. Likewise, errors of internal standard addition can also be eliminated. All these common errors, in addition to others are minimized by automating sample preparation.

Extraction technique	Dynamic range	R^2	Curve weighting	%Accuracy range (*N=3*)	%RSD range (*N=3*)
PPT (Sirocco)	2–500 ng/mL	0.993	$1/x^2$	92.4–105.5	0.2–3.7
PPT with PL removal (Ostro)		0.997		97.6–103.4	0.2–14.9
SSLE		0.993		86.0–109.1	0.4–6.1
RP SPE (HLB)		0.996		93.4–109.9	0.7–2.4
RP SPE with PL removal (Oasis PRiME HLB)		0.996		87.9–110.8	0.3–3.8
Mixed mode SPE (MCX)		0.996		94.2–109.7	0.2–3.7

Table 3. Performance of calibration curves extracted by Andrew+ Pipetting Robot configured with the Extraction+ Connected Device (N=3). Curve fits, %accuracy and %RSD are listed for each sample preparation technique.

Quantitative quality control results						
	Low QC		Mid QC		High QC	
	Mean (N=3) % Accuracy	% RSD	Mean (N=3) % Accuracy	% RSD	Mean (N=3) % Accuracy	% RSD
PPT (Sirocco)	92.6	1.0	97.9	1.8	95.0	0.3
PPT with PL removal (Ostro)	98	6.2	99.9	0.8	94.6	1.3
SSLE	97.0	1.4	96.1	1.8	90.8	0.6
RP SPE (HLB)	100.9	0.8	99.1	3.9	89.9	4.2
RP SPE with PL removal (Oasis PRiME HLB)	103.0	2.0	99.4	0.5	93.3	0.3
Mixed mode SPE (MCX)	105.7	3.0	103.7	2.3	96.1	1.0

Table 4. Accuracy and precision results from QC samples and extracted using Andrew+ configured with Extraction+ Connected Device.

Conclusion

This application highlights the successful use of the Andrew+ Pipetting Robot configured with the Extraction+ Connected Device for fully automated sample preparation for a variety of common bioanalytical sample preparation techniques. In addition, available methods in the OneLab library can be used to minimize protocol development time. Except for some minor interventions, all aspects of the extraction techniques, including pipetting, mixing, vacuum profiles, waste handling, and sample dilutions were fully automated. Recovery and matrix effect data show that Andrew+ and Extraction+ have the versatility and flexibility to execute a variety of sample preparation techniques, enabling rapid method evaluation and profiling to determine a fit-for-purpose method. The quantitative results demonstrate the excellent precision achieved by this automated system, with most %RSDs in the single digits.

The ability to automate the bioanalytical sample preparation workflow has several advantages. The reliability and reproducibility enable consistent results that often equal or exceed those achieved by manual preparation. Other sources of error, such as sample transposition, addition of incorrect reagents, spiking errors, and technique sensitive steps such as vacuum elution are nearly eliminated, reducing the risk of failed batches and increasing productivity. Eliminating much of the manual pipetting tasks in the lab also helps reduce the risk of repetitive

stress injuries for laboratory scientists. Finally, the time saved enables scientists in the laboratory to focus on other areas rather than repetitive manual tasks.

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