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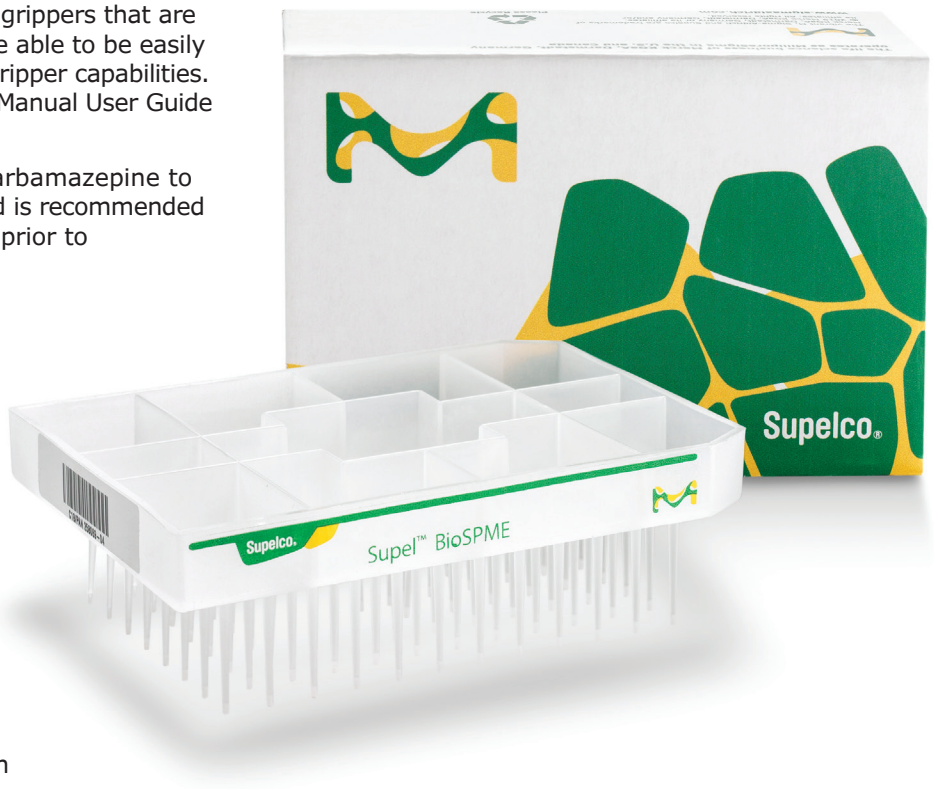
Supel™ BioSPME C18 96-Pin Devices

User Guide for Automation Workflow

For Plasma Protein Binding Determination and Free Analyte Analyses

This guide offers an outline to perform the Supel™ BioSPME C18 96-Pin Device technique on a Hamilton® STARlet system, however, these devices are maneuvered via grippers that are common on other instruments and should be able to be easily maneuvered on any automation robot with gripper capabilities. For manual workflows, please download the Manual User Guide on our website.

The following guide uses the example of carbamazepine to demonstrate the extraction methodology and is recommended as a starting point to validate the workflow prior to implementing on a broader scale. In this workflow, the extraction of analytes is performed out of both buffer and spiked plasma samples, with protein binding calculated afterwards using the extracted amounts.



Necessary Equipment

1. A Hamilton® STARlet robotic system equipped with a deck layout to accommodate various consumables, specifically: four 96-well plates (one conditioning plate, a wash plate, a sample plate, and a desorption plate), three liquid reservoirs, a Supel™ BioSPME C18 96-Pin Device, two different sized pipette tips, and a heated shaker.

   Heated shaker for Hamilton® robots:
   199034 HHS2 3.0 flat bottom heater/shaker

   Shaker plate that fits CoStar™ plates: 199026 Flat adapter kit, usually comes with recommended shaker.

2. Round bottom or conical 96-well plate with a maximum depth of the wells at 31 mm (example listed under Recommended Consumables section).

3. Adapter to position pin-tool into the robotic deck (Catalogue No. 59686-U)

List of Recommended Consumables

1. Supel™ BioSPME C18 96-Pin Devices, 1 pack 59680-U OR Supel™ BioSPME C18 96-Pin Devices, 10 pack 59683-U
2. Positioning Adapter (for Automation of Supel™ BioSPME C18 96-Pin Devices), 1 pack 59686-U
3. Phosphate Buffer Solution (Catalogue No. P5368)
4. Human plasma, K2EDTA, pooled gender
5. Carbamazepine solution 1 mg/mL methanol (Catalogue No. C-053)
6. Carbamazepine-d10 solution 100 µg/mL methanol (Catalogue No. C-094)
7. Nunc, 1 mL round well, 96-deep well plate (Catalogue No. P8241) for condition and wash steps
8. Eppendorf, 600 µL round well, 96-well plate (Catalogue No. EP951031861) for sample desorption
9. CoStar™ plate, 320 µL V-bottom well, 96-well plate (Catalogue No. CLS3357-100EA) for sample extraction at 200 µL volumes
10. 2-Propanol, LC/MS grade (Catalogue No. 1.02781)
11. Water, LC/MS grade (Catalogue No. 1.15333)
12. Methanol, LC/MS grade (Catalogue No. 1.06035)
13. Zone-Free Sealing Film (Catalogue No. Z721646-50EA) or equivalent
14. SealPlate film (Catalogue No. Z369659-100EA) or equivalent
15. Eppendorf Plate Lid (Catalogue No. 0030131517-80EA)

Standards and Solutions

1. **Plasma:** Spike human plasma with carbamazepine at 100 ng/mL (or at 0.42 µM). Mix spiked plasma on vortex mixer at the mixing speed of at least 1250 rpm for 30 seconds. Equilibrate spiked plasma sample at 37 °C for 60 minutes under mild agitation (shaking) to reach the equilibrium between protein-bound and unbound analyte. It is advisable to equilibrate plasma as one volume and aliquot later into the 96-well plate.
2. **PBS solution:** Dissolve 1 packet Phosphate Buffered Saline in 1 L water. Solution may be kept up to 1 month in refrigerator.
3. **Buffer:** Prepare phosphate buffer spiked with carbamazepine at 100 ng/mL. It is recommended to follow the same equilibration as the plasma to ensure homogenous distribution of analyte throughout the buffer.
4. **Desorption solution:** Prepare a mixture of 80% methanol, 20% LC/MS grade water (v/v) containing internal standard carbamazepine-d10 at 10 ng/mL. The deuterated analog is used to correct for instrument drift and/or sample evaporation.

Extraction Method Overview

1. **Conditioning—**Program the robot to immerse the pin device into the conditioning plate (100% 2-propanol) for 20 minutes without shaking.

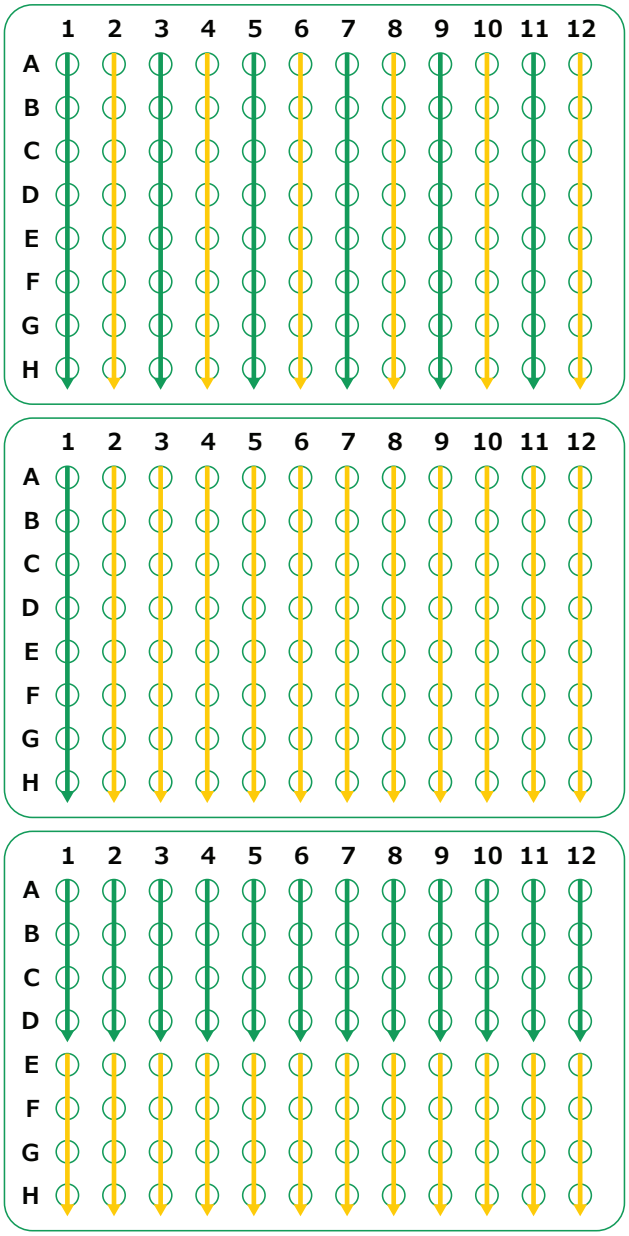
   The coated adsorbent particles need to be pre-conditioned using organic solvent. 100% 2-propanol should be used for conditioning step without shaking. For conditioning and ensuing steps, it is important that the volume used allows for the pins to be submerged.
2. **Wash (Water rinse)—**Immerse the pin device into the wash plate (100% water) for 10 seconds. Ensure there is enough water in the wells that the coated portion of the tip does not dry.
3. **Extraction—**This step is performed under agitation at 1200 rpm. Both matrix (plasma) and buffer samples need to be at the same temperature during extraction. Buffer is used for calibration of the amount extracted by the Supel™ BioSPME C18 96-Pin Devices. A 200 µL volume is recommended per well for extraction. Program the robot to immerse the pin device into the extraction plate for 15 minutes at 37 °C at 1200 rpm.
4. **Wash (Water rinse)—**Program the robot to immerse the pin device into the wash plate (100% water) for 60 seconds. It is important that the pin device is submerged into water and do not dry.
5. **Desorption—**Program the robot to immerse the pin device into the desorption plate (80:20 methanol:water and internal standard) for 20 minutes without shaking.

Desorption can be done using 50–200 µL volumes. The times for desorption will need to be decreased for lower volumes to mitigate evaporation. Care needs to be taken to avoid evaporation of the desorption solvent as it contains 80% methanol. It is recommended to use a plate cover during the extraction procedure and remove the plate cover prior to the desorption step. The generic Hamilton® program allows for desorption using 200 µL volumes.

Example Layouts for Extraction Plate

96-well Extraction Plate—Pipette 200 µL aliquots of either buffer or plasma per well, using separate columns for each; according to one of the illustrations below. We recommend each test be performed in triplicate to minimize the risk of erroneous data. Once the extraction plate is loaded, place it on the heated shaker to equilibrate at 37 °C while shaking at 300 rpm (RCF = 0.2; r = 1.5 mm) until the pin device is attached.

Basic Sample Layout Strategy

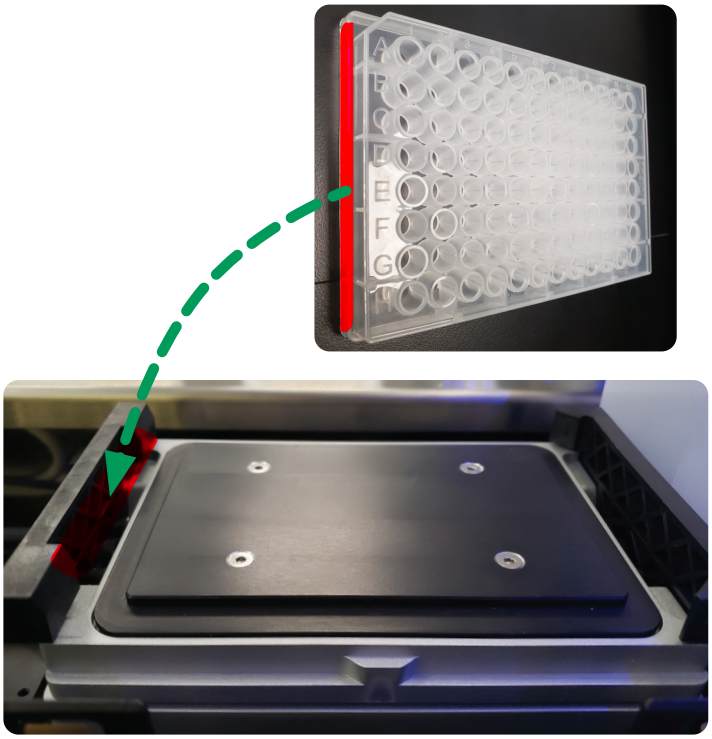


Green = buffer
Yellow = plasma

Robotic Automated Method

The Hamilton® robot (or other automation robot) will use paddle grippers to manipulate the Supel™ BioSPME C18 96-Pin Devices and move them between conditioning, wash, extraction (sample) and desorption plate positions. The grippers will hold the pin device immersed in the 96-well plates for static/non-shaken steps and also immersed in the agitated/shaken plate for extraction. The position of the pin device above the extraction well plate needs to be set with respect to the type of well plate used for extraction. The program described here was developed for using pin devices with 200 µL extraction sample volumes in the recommended CoStar™ plate. When other sample volumes or 96-well plates are used, the height of the pin device inside the wells needs to be adjusted accordingly. Lower desorption volumes down to 50 µL can also be used in PCR-type conical well plates, however, the program needs to be changed to reflect this new volume and type of well plate.

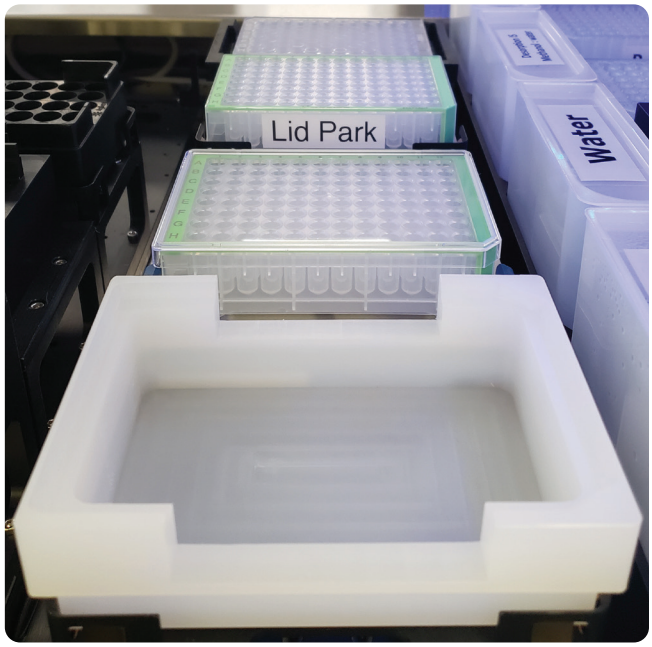
1. Load the 96-well extraction plate (CoStar™ plate) onto the Hamilton® heated shaker. The extraction plate should already contain buffer and plasma solutions.

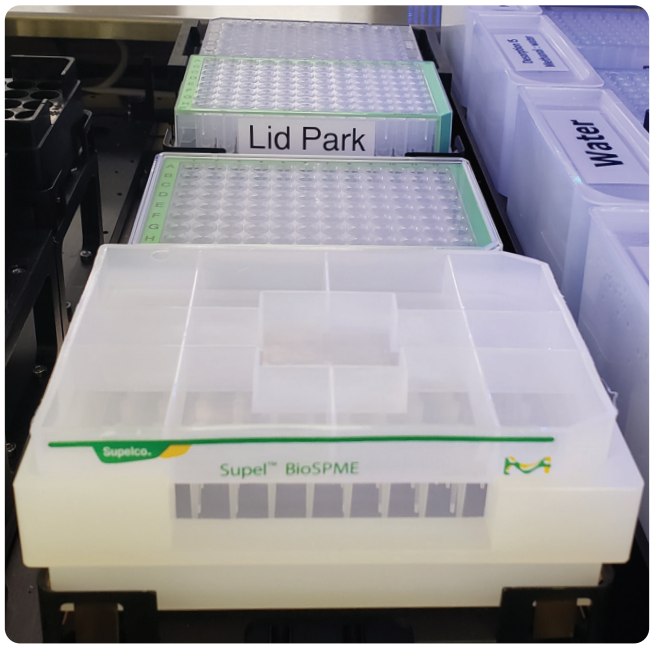


Place the well plate skirt (left edge) up against the flat left side rail of the heated shaker approximately centered (forward/back)

2. Place the conditioning, wash, and desorption well plates in their designated positions. In addition, place the cover (Eppendorf Plate Lid) on the desorption plate to prevent evaporation of desorption solution.

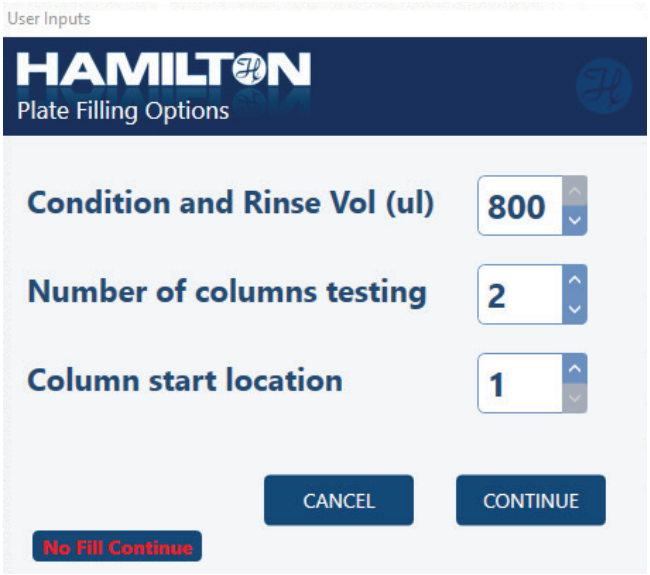
3. Fill solvent reservoirs with conditioning, wash, and desorption solvents.





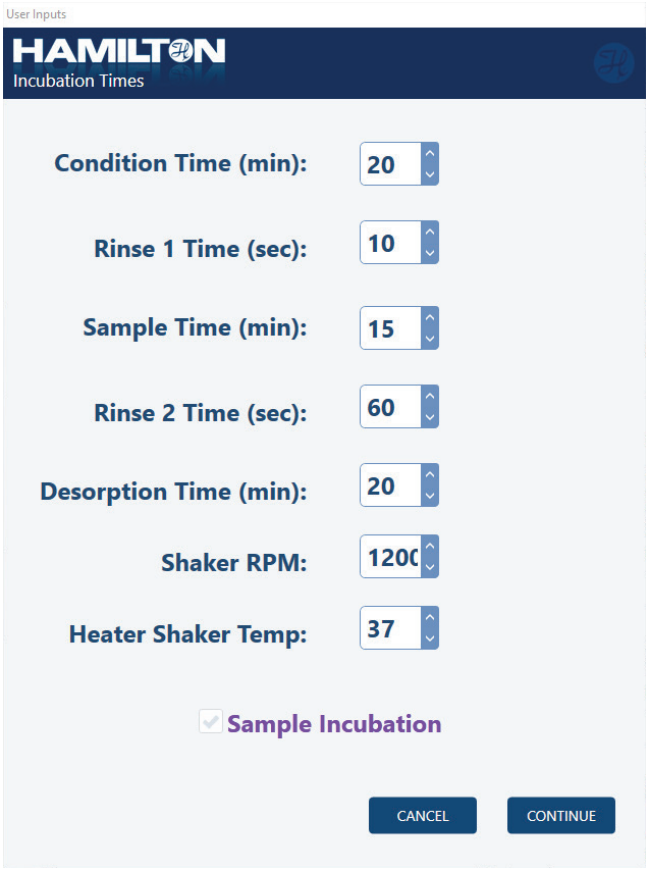
4. Load the Supel™ BioSPME C18 96-Pin Device into the adapter, the adapter should be positioned in place of 96-well plate.
5. Start automated program from the Hamilton® dashboard or another program to use with Supel™ BioSPME C18 96-Pin Devices. The generic program can be downloaded by following the link on our webpage.

6. The program will present the following input screens:

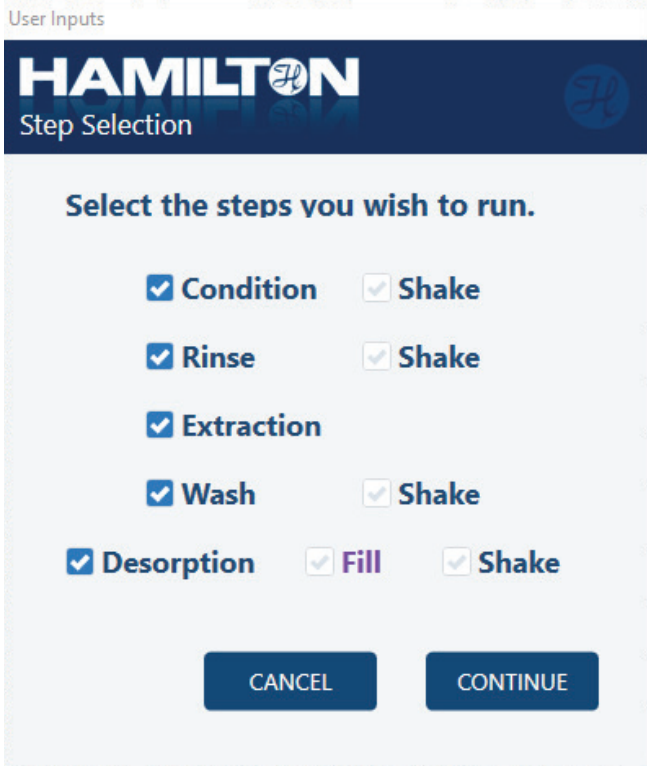


This allows for changing the conditioning and wash/ rinse volumes, the number of consecutive columns to be tested, and the location of the first column.

“No Fill Continue” allows you to opt out from having the Hamilton® robot fill the condition and wash plates. This is available if non-consecutive columns or partial columns are used and thus will require manual filling.



This screen allows for setting the timing, shaker speed, and shaker temperature for processing.



This screen allows the selection of which steps would include the option of shaking for the specified step. Shaking on the extraction step is always enabled.

There is also the option to fill the desorption plate with 200 µL in each well of desorption solution from the reservoir corresponding to the number of columns and start location selected in the first user input screen.

Note: If non-consecutive columns or partial columns are used, please fill the desorption plate manually as with the conditioning and wash plates.

7. When the program is started, the heater will begin to heat the extraction plate. The heater needs to reach the set temperature (37 °C) in order for the program to start running.
8. Remove the covers of the bulk solutions before hitting "continue." The program will initiate pipetting of solutions into the conditioning, wash, and desorption well plates if selected before running the Supel™ BioSPME C18 96-Pin Device method (as described in the Extraction Method Overview and to the right).

Robot grips pin tool from its parked position

Pin tool is taken to the conditioning plate for 20 min

Pin tool is taken to the water wash for 10 seconds

Pin tool is taken to the extraction plate and shaken for 15 minutes while extracting

Pin tool is taken to the water wash for 60 seconds

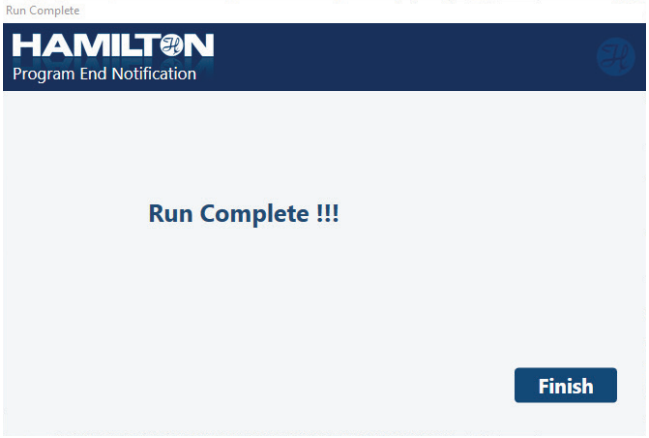
Pin tool is taken to the parking position

The plate cover of the desorption plate is taken off by the grippers

The pin tool is gripped from its parked position and taken to the desorption plate for 20 minutes

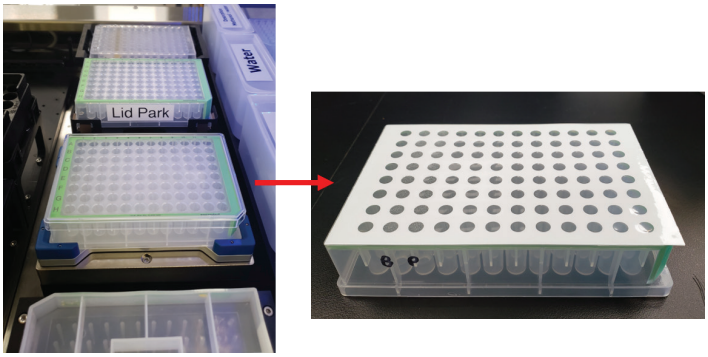
The pin tool is returned to its parking position

The plate cover is returned to the desorption plate

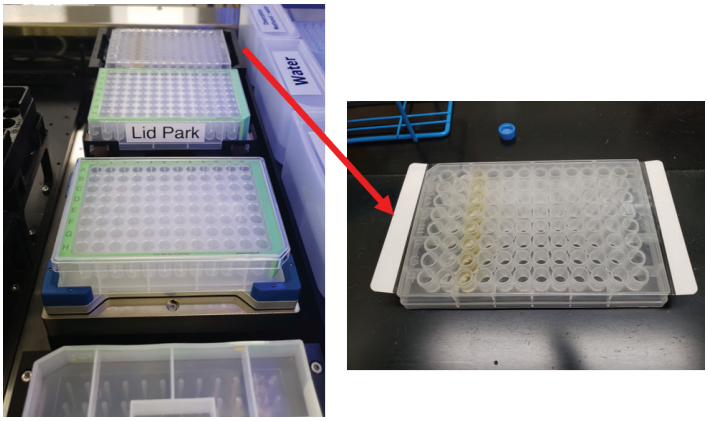


When the run is complete, click “Finish” to end the program.

9. Wait until the home screen appears to open the sash to remove the desorption plate. Opening the sash early will result in an instrumental error.



10. Remove the extraction (sample) plate from the deck and seal with sealing film before biohazardous disposal. Supel™ BioSPME C18 96-Pin Devices can be disposed into regular trash after desorption using organic solvent.



LC-MS Analytical Method

Prepare external calibration standards of carbamazepine using the desorption solution (80:20 methanol:water containing 10 ng/mL carbamazepine-d10) to cover a range from 1 to 50 ng/mL. These will be injected along with the extracted samples and used to determine the mass extracted.

Column: Ascentis® Express C18 column (5 cm x 2.1 mm, 2.7 µm)

Mobile phase A: 5 mM ammonium formate with 0.01% formic acid in 95:5 water:acetonitrile

Mobile phase B: 5 mM ammonium formate with 0.01% formic acid in 5:95 water:acetonitrile

Column Temp: 40 °C

Inj. Vol: 2 µL

Flow Rate: 0.4 mL/min

Gradient: MS/MS parameters (from Sciex 6500 system)

ESI (+)

Time	0	0.5	3.0	4.0	4.1	6.0
%B	10	10	90	90	10	10

Analyte	Precursor	Product	Dwell (msec)	DP (volts)	EP (volts)	CE (volts)
Carbamazepine	237.1	194	75	35	7	29
Carbamazepine-d10	247.1	204.1	75	35	7	29

Determination of Protein Binding

The percent free or percent unbound is determined in **Eq. 1**:

Eq 1. $$\text{Percent Free} = \text{Free Fraction } (F_U) \times 100$$

where concentration free represents the unbound concentration of the analyte in the matrix (in this case plasma), and concentration total represents the total concentration of analyte. The amount extracted is independent of units and can be calculated using preferred quantities (e.g. nanograms or moles) M_{free}, and extraction volume of plasma, V_{plasma}. The concentration of analyte in the desorption solution is quantified by an external calibration curve, and if the desorption volume is equal to the plasma and buffer extraction volumes, the concentration from desorption will be equal to the extracted concentration as shown in **Eq 2.**

Eq 2. $$\text{concentration extracted from plasma, } P = \frac{M_{(E,Plasma)}}{V_{plasma}}$$

Eq 3. $$\text{concentration extracted from buffer, } B = \frac{M_{(E,Buffer)}}{V_{Buffer}}$$

The percent bound can be determined from the extracted concentrations as shown in **Eq 6**.

Eq 4. $$\text{Percent Bound} = 100\% - \text{Percent Free}$$

Eq 5. $$\text{Percent Bound} = \text{Bound Fraction } (F_B) \times 100\%$$

Eq 6. $$\text{Percent Bound} = \frac{(B - P)}{B} \times 100\%$$

Eq 7. $$\text{Percent Free} = \frac{P}{B} \times 100\%$$

where B and P, represents the amount extracted from buffer (B) or plasma (P). In cases where depletion of compounds from plasma is pronounced upon Supel™ BioSPME C18 96-Pin Device extraction (extraction exceeded 5% of total spiked analyte), a correction to the calculated Bound Fraction is required as described below:

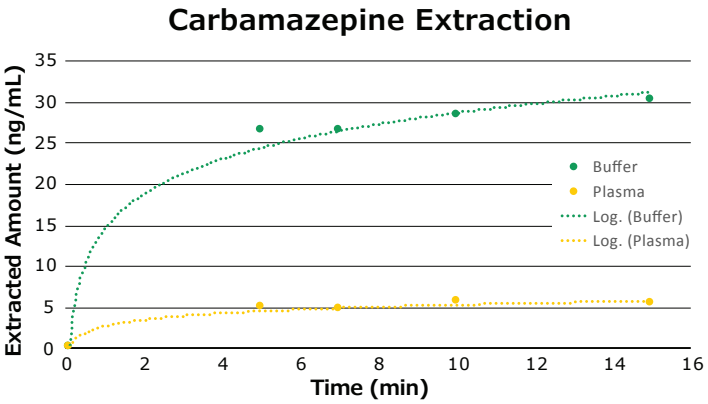
Eq.8 $$\text{Bound Fraction } (F_B) = \frac{[P^0 - \frac{[(B^0 - B) \times P]}{B} - P]}{P^0 - P}$$

where B and P, represent the respective amounts extracted from buffer, B, and plasma, P. P^0 and B^0 represent the concentration at which the plasma (P^0) and buffer (B^0) were initially spike or the total concentration. **Eq. 8** accounts for the concentration in solution after extraction on the fiber: the depletion of the analyte from sample. **Eq. 6** and **Eq. 7**, do not take this consideration into account. However, they provide accurate values when the extracted amount from plasma is less than 5% of the total spiked amount.

Troubleshooting and FAQ

1. How long is the extraction time?

The recommended extraction time is 15 minutes for 200 µL volume under shaking at 1200 rpm on an orbital shaker with orbital radius with 1–2 mm. This extraction time can vary for different compounds as the extraction kinetics will vary depending on compound properties. It is important to achieve equilibrium between the analyte in solution and analyte adsorbed into coating phase or at least get close to such equilibrium. This is illustrated by using extraction of spiked carbamazepine in 200 µL in buffer and plasma where 15 minutes is used as the extraction time and the system is sufficiently close to equilibrium conditions.



2. Which desorption volumes can be used in the Supel™ BioSPME C18 96-Pin Device procedure?

During desorption, it is recommended to use a mixture of water and organic solvent(s) to desorb analytes from the coating. Care needs to be taken to avoid evaporation of the desorption solution. Although a higher volume of desorption solution (200 µL) will help to maintain reproducibility by preventing evaporation, note that this higher volume may cause a decrease in sensitivity. Use a cover for the desorption well plate prior to desorption step to prevent evaporation. If smaller volumes are required to be used for desorption, e.g., when pre-concentration of analyte is necessary for the detection sensitivity, 50 µL volumes can be used in a PCR-type plates. For 50 µL desorption volumes 5 minutes desorption time can be sufficient in comparison to 20 minute desorption time when using 200 µL volumes.

3. Is it necessary to use internal standard?

Internal standard in the desorption solvent can be used to correct for variabilities due to evaporation, injection volume and mass spectrometric detection. While an internal standard does not undergo extraction, it can point to other sources of uncertainty in the method.

4. How to mitigate the effects of non-specific binding of compounds to plastic well plates?

Hydrophobic compounds (with logP of 3.5 and above) can undergo non-specific binding to the plastic well plates. Some of the positively charged compounds were also found to have lower extraction efficiencies from plastic polypropylene plates in buffer. This can significantly change the extracted amounts from buffer and result in introduction of errors into protein binding values found by BioSPME. Any additives, including surfactants, can be extracted by the Supel™ BioSPME C18 96-Pin Device and contaminate the LC-MS instrumentation. It was found that using glass-lined plates resolved the non-specific binding issues for most analytes studied. These plates are commercially available from Thermo-Scientific, for example.

5. Can a BioSPME method provide protein binding for analytes with different charge at physiological pH?

Use of C18 adsorbent coating is appropriate for analytes that are neutral, and have a single positive or negative charge at physiological pH. The analytes that carry multiple charges may not extract efficiently into the C18 based coating and therefore, for these analytes BioSPME-C18 may not be an appropriate method for protein binding determination.

6. Can a BioSPME method provide protein binding for analytes with different molecular weights?

The C18 adsorbent used in the Supel™ BioSPME C18 96-Pin Device coating has the pore size of 180 Å. The coating was tested for analytes up to 1200 Da and found to be acceptable for extraction providing good quantitative extraction from both buffer and plasma using the suggested generic methodologies. Some analytes with higher molecular weight can exhibit slower extraction kinetics and their equilibration would take longer time than suggested

generic 15 minutes. One such analyte was cyclosporine with equilibration time of more than 4 hours. The larger analytes which also carry multiple charges at physiological pH may not be efficiently extracted into the coating of the hydrophobic C18 adsorbent.

7. How can I adapt method for my analyte?

Method parameters that can be changed include: sample volume, extraction time, volume of desorption solution, desorption time, buffer concentration. Reaching equilibrium extraction conditions and full desorption of the analyte will need to be confirmed for method changes.

8. How can I achieve less than 15% reproducibility on extraction?

Please, review the following steps in the extraction and detection method to insure good extraction reproducibility:

- Conditioning of the Supel™ BioSPME C18 96-Pin Device is performed as required to activate the adsorbent
- Coating is not allowed to dry between conditioning and extraction and between extraction and wash steps, transition of the pin tool between the method steps is done within 10–20 seconds
- The levels of solvents and the sample in the wells is high enough to fully submerge the coating
- Pin device is levelled across the well plate and the grippers work correctly
- The analyte in buffer solution was tested for the non-specific binding to the plastic plates. If non-specific binding is exhibited by analyte, the use of glass-coated plates is recommended
- The pH of the sample is the same across multiple extractions
- The temperature of the samples under extraction is well-controlled using heating adapters, the temperature across the well-plate can be checked using thermocouple when the setup of the method is performed
- The sample is well-equilibrated prior to extraction, we recommend using at least 1 hour incubation time
- The extraction time is sufficiently long to achieve equilibrium but not too long to allow the extraction competition from sample lipids. Too short and too long extraction times can also result in lower accuracy of the protein binding values
- The desorption time is sufficiently long to allow full desorption of the analyte from the coating into the organic solvent
- LC-MS instrument reproducibility is checked and found to be acceptable (at below 3–4% CV) when sample in buffer is injected multiple times. LC-MS detection is above lower limit of quantitation.

Additional information on development of Supel™ BioSPME methods as well as comparison data with rapid equilibrium dialysis is available on our website, **www.sigmaaldrich.com/biospme**.

To place an order or receive technical assistance

please visit, **www.SigmaAldrich.com**

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