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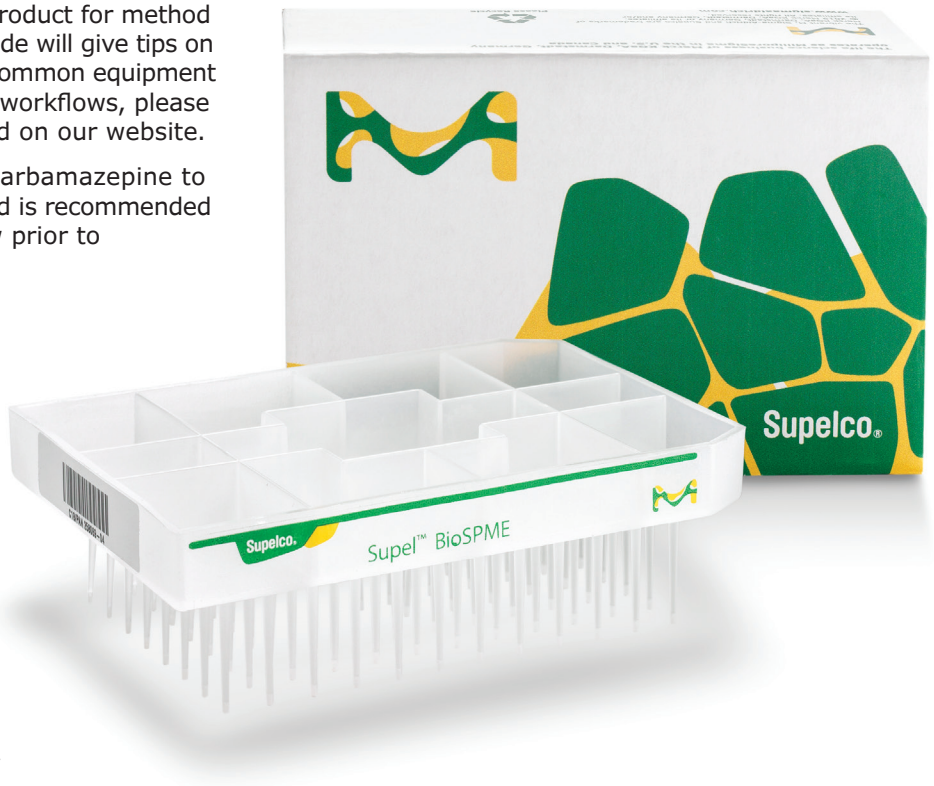
Supel™ BioSPME C18 96-Pin Devices

User Guide for Manual (Non-Automation) Workflow

For Plasma Protein Binding Determination and Free Analyte Analyses

The Supel™ BioSPME C18 96-Pin Device was developed to be used on robotic systems, but we recognize necessity for some customers to be able to manually use this product for method development and validation. This manual guide will give tips on using the pin device in a basic set up using common equipment found in most laboratories. For automation workflows, please download the Automation User Guide found 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 heated shaker with the capability of fitting a 96-well plate with an attached Supel™ BioSPME C18 96-Pin Device. A heating adapter that conforms to the 96-well plate is also required to evenly distribute heat to the 96-well plate. The shaker should have the ability to provide shaking speeds of 300–1200 rpm (RCF = 0.02–2.4; r = 1.5 mm) with a small orbital radius of 1–2 mm.
2. Multi-channel Pipette (recommended but can be accomplished with single channel pipette).
3. Round or conical 96-well plates with a maximum depth of the wells at 31 mm (examples listed under Recommended Consumables section).

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. Phosphate Buffer Solution (Catalogue No. P5368)
3. Human plasma, K2EDTA, pooled gender (BIOIVT part no. HumanPLK2PNN)
4. Carbamazepine solution 1 mg/mL methanol (Catalogue No. C-053)
5. Carbamazepine-d10 solution 100 µg/mL methanol (Catalogue No. C-094)
6. Axygen®, 560 µL conical wells, 96-well plate (VWR 89182-902, Axygen P-DW-500-C) or Eppendorf, 600 µL round wells, 96-well plate (Catalogue No. EP951031861) or plate with equivalent well depth and plate height
7. 11 mm Thermogreen® LB-2 Septa (Catalogue No. 20654) —for spacing the pin device and the 96-well plates
8. 6 mm Thermogreen® LB-1 Septa (Catalogue No. 20668), cut the height in half, or 6 mm Thermogreen® LB-2 Septa (Catalogue No. 20608) can be used for indexing the pin device to the 96-well plates
9. 2-Propanol, LC/MS grade (Catalogue No. 1.02781)
10. Water, LC/MS grade (Catalogue No. 1.15333)
11. Methanol, LC/MS grade (Catalogue No. 1.06035)
12. Zone-Free™ Sealing Film (Catalogue No. Z721646-50EA) or equivalent
13. A shaker such as MultiTherm™ shaker with heating (Catalogue No. Z755753/Z755761)
14. Shaker heating block such as Catalogue No. Z755796.

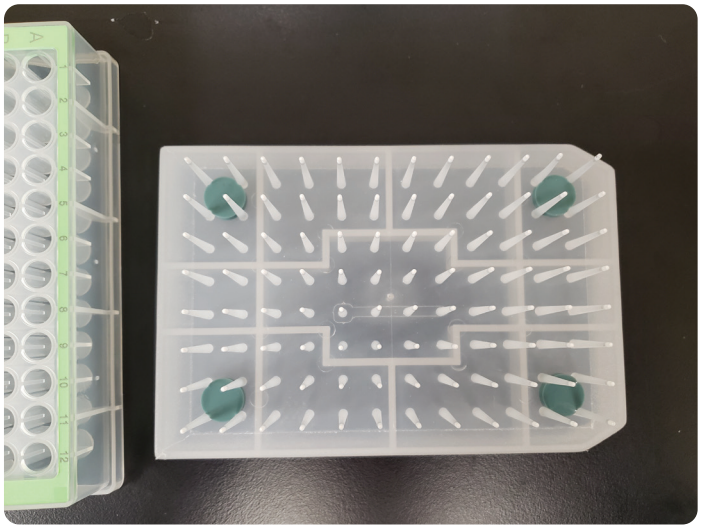
Standards and Solutions

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.

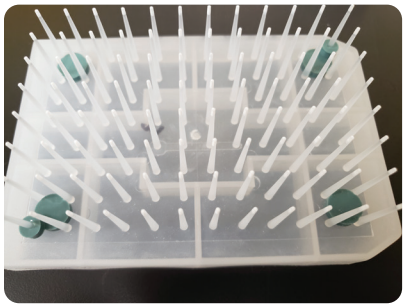
Preparation of Supel™ BioSPME C18 96-Pin Devices and Well plates

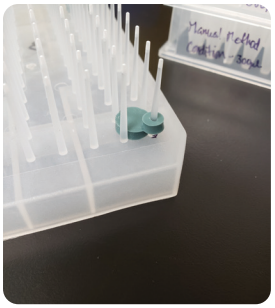
Prior to running the extraction method, there are some modifications that will need to be done for adequate fitment of the 96-Pin device with the 96-well plates to perform the manual workflow.

1. **Pin device spacing—**Place septa on the pin device as shown below to provide the appropriate height spacing. Placement can be changed dependent upon which columns or rows are being used.



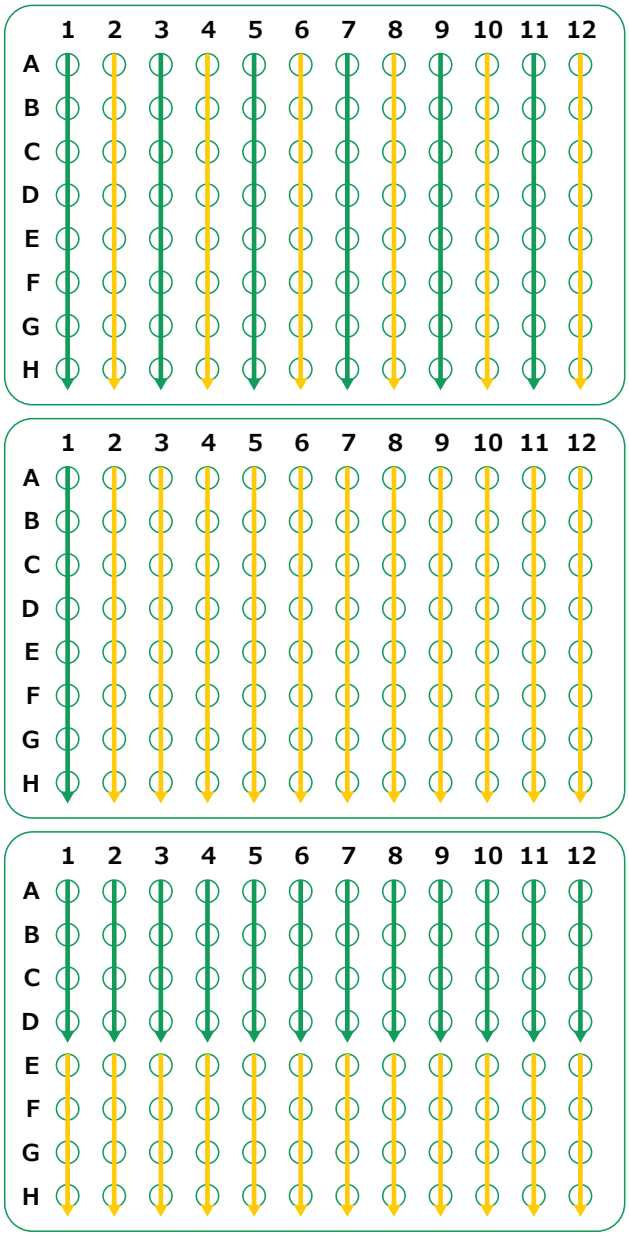
2. **Pin device indexing—**place a small (6 mm O.D.) septa piece (which has been cut in half) on two corner pins to maintain pin position within the xy-plane of the wells. Note: these pins cannot be used for analysis.





3. **96-well Conditioning Plate—**Pipette 400 µL aliquot of conditioning solution (2-propanol) into the wells being used in the 96-well conditioning plate.
4. **96-well Wash Plate—**Pipette 500 µL aliquot of wash solution (water) into the wells being used in the 96-well wash plate.
5. **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 on the following page. 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.

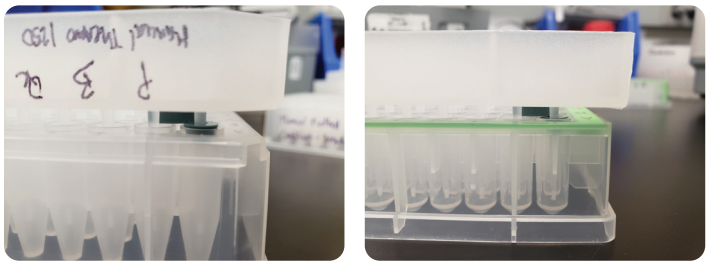
Basic Sample Layout Strategy



Green = buffer
Yellow = plasma

Extraction Method Overview

For all steps in the extraction method, place the pin device so that the spacers and indexing septa are between the pin device and 96-well plate, as shown below:



1. Place the Extraction well plate on the heated shaker and shake at 300 rpm for at least 20 minutes.
2. Conditioning—Immerse the Supel™ BioSPME C18 96-Pin Device into the conditioning plate (100% 2-propanol) for 20 minutes without shaking. This can be done simultaneously with step 1 and does not require the heat or shaker.

   The coated adsorbent particles need to be pre-conditioned using organic solvent; 100% 2-propranolol is recommended. A volume of 400 µL is recommended per well as it is important that the pin tip does not dry between the conditioning and extraction steps.

   Toward the end of the conditioning step, turn off the shaker that contains the extraction plate in order to make transfer after the next step easier. The extraction plate has been equilibrated on the shaker at this point.

3. Wash (water rinse)—Immerse the pin device into the wash plate (100% water) for 10 seconds. A volume of 500 µL volume is recommended as it is important that the pin tip does not dry.

4. Extraction—This step is performed under agitation at 1200 rpm (RCF = 2.4; r = 1.5 mm). 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 BioSPME pin devices. A 200 µL volume is recommended per well for extraction for 15 minutes at 37 °C. Transfer the pin device to the extraction plate with the indexing pieces in place. It is suggested that tape be used to help secure the pin device to the well plate. Set the shaking speed to 1200 rpm and start the shaker.

5. Wash (water rinse)—Immerse the pin device into the wash plate (100% water) for 60 seconds. Make sure that the pin device is submerged into the water. It is important that the pin tip does not dry.

6. Desorption—Immerse the pin device into the desorption plate (80:20 methanol:water with internal standard) for 20 minutes without shaking.

   Desorption can be done using 50–200 µL volumes. 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.

   Mixing is important prior to analysis to ensure the homogeneity of the solution. This can be critical depending upon the design of the well plate being used. If using conical well plates, it is suggested to use pipette mixing as additional shaking has been found to be ineffective at this point. Even if using round well plates, it is recommended if analysis is performed immediately after the desorption step that the plate be shaken for 20 seconds at 1000 rpm.

LC-MS Analytical Method

Prepare an external calibration of carbamazepine in 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:

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

MS/MS parameters (from Sciex 6500 system)

ESI (+)

Analyte	Precursor	Product	Dwell (msec)	DP (volts)	EP (volts)	CE (volts)
Carbamazepine	237.1	194.0	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. $\text{Bound Fraction } (F_B) = 1 - \text{Free Fraction } (F_U)$

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

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. What type of shakers can be used in Supel™ BioSPME C18 96-Pin Device extractions?

A shaker with heating capability should be used for the extraction step. The shaker should have an orbital radius of 1–2 mm and provide even heating across all 96 well of the well plate (such as with heating blocks). It is not recommended to use shakers with the orbital radius exceeding the well dimensions as efficient shaking will not be achieved.

2. Which agitation speeds can be used in Supel™ BioSPME C18 96-Pin Device extractions?

Fast extraction of the analyte from both buffer and plasma solution using Supel™ BioSPME C18 96-Pin Devices is only possible under efficient agitation conditions. This is required to achieve the equilibrium between analyte extracted onto the device and analyte remaining in the sample. The equilibrium can generally be achieved during the recommended extraction time of 15 minutes when agitation at 1200 rpm is used and the orbital radius is 1–2 mm. Lower agitation speeds may require longer extraction times and result in increased extraction variability. Faster agitation speeds can result in the formation of a vortex in the middle of the well and an insufficient sample level for extraction of the analyte onto the pin device. Please, see more information of how to convert rpm to RCF(g) using the link to our web-site.

www.SigmaAldrich.com/technical-documents/articles/biofiles/centrifugation-basics.html

3. Can extraction be done without agitation (statically)?

Static extraction or the extraction without sample agitation does not provide appropriate conditions to achieve fast equilibration of analyte between Supel™ BioSPME pin and sample. Only using the concentrations measured at equilibrium conditions can result in accurate determination of free fraction and free analyte concentration. Achieving equilibrium under static conditions will take more than 60 minutes and can take up to several hours depending on the analyte. Extended time period will lead to a change of plasma pH and inaccurate determination of protein binding. Extraction without agitation is not recommended.

4. What type of well plates can be used in Supel™ BioSPME C18 96-Pin Device extractions?

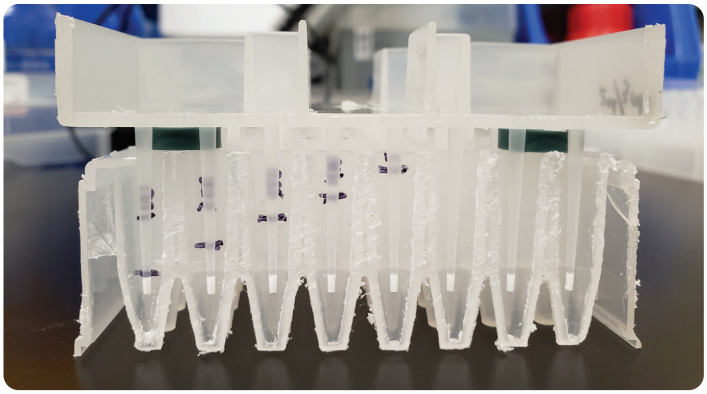
Three major shapes of well plates are available from multiple vendors. Round-well plates are made for sample agitation and should be used for Supel™ BioSPME C18 96-Pin Device extractions. Conical-shaped wells are made to minimize the sample volumes. These can be utilized if small sample volumes are required. Samples in conical wells need to undergo effective homogenization step prior to analysis, such as via the use of pipetting. Square-shaped well are usually made for sample storage and are often light-permeable for studies. Square-shaped well should not be utilized for Supel™ BioSPME extractions C18 96-Pin Device as they provide non-efficient extraction conditions.

5. What are recommended desorption volumes for the Supel™ BioSPME C18 96-Pin Device procedure?

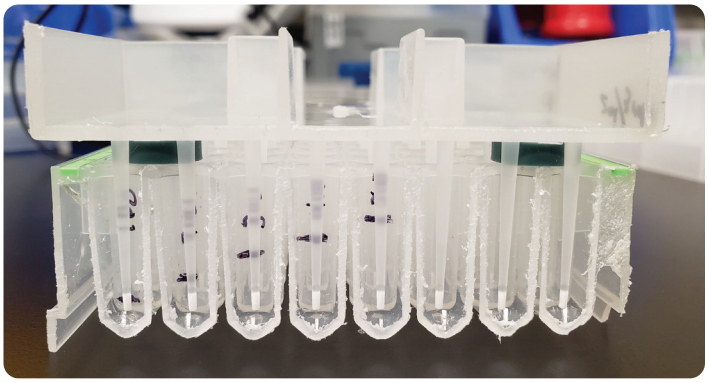
During desorption, it is recommended to use a mixture of water and organic solvent(s) to desorb analytes from the coating. Care needs to be taken to avoid evaporation of the desorption solution. Although higher volumes of desorption solution will help to maintain reproducibility, note that higher volumes of desorption solution will also decrease sensitivity. Make sure to use a cover for the desorption well plate prior to the 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 96-well plate. For 50 µL desorption volumes, a 5 minute desorption time can be sufficient in comparison to the recommended 20 minute desorption time when using 200 µL volumes.

6. What are recommended sample volumes for Supel™ BioSPME C18 96-Pin Device extractions?

During Supel™ BioSPME C18 96-Pin Device extractions, the volume of the extraction phase on the pin device should be much smaller (e.g. factor of 100–1000) than the sample volume. This is why procedures to be used with Supel™ BioSPME C18 96-Pin Devices recommend using no less than 200 µL samples. Limited success can be achieved using 100 µL samples. If 100 µL samples are desired, the extraction time length needs to be adjusted down due to more efficient agitation of smaller volume from 15 minutes (used for 200 µL samples) to 5–10 minutes. If smaller samples need to be analyzed, a dilution is recommended. Example of positioning pins into the well plates with different sample volumes are shown below.



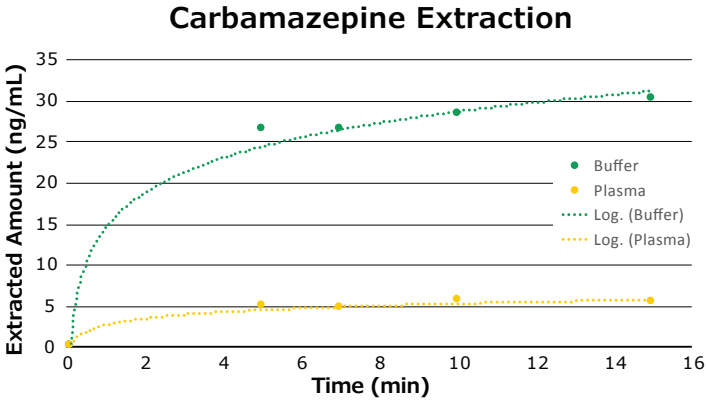
Cross-section: Axygen® conical well plate with spacer (green)
Markings are for 100 µL through 500 µL in increment of 100 µL (left to right)



Cross-section: Eppendorf round well plate with spacer (green)
Markings are for 100 µL through 500 µL in increment of 100 µL (left to right)

8. 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.



9. Can diluted samples be used for protein binding determination?

If d is the dilution factor (for 1:1 buffer:plasma dilution d=2, for 1:4 dilution d=5) and $\%Bound_{apparent}$ is the apparent protein binding found in diluted plasma sample, then

Eq 9. $$\%Bound = \frac{100 \times d \times \%Bound_{apparent}}{100 + \%Bound_{apparent} \times (d - 1)}$$

We do not recommend to dilute plasma more than a factor of 5 in the protein binding test.

10. 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 internal standard does not undergo extraction, it can point to other sources of uncertainty in the method.

11. 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 BioSPME and contaminate the LC-MS instrumentation. It was found that using glass-lined plates resolved the non-specific binding issues for most analytes studied during BioSPME development. These plates are commercially available from Thermo-Scientific, for example.

12. 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.

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

The C18 adsorbent used in the Supel™ BioSPME C18 96-Pin Devices 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.

14. 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.

15. 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 Supel™ BioSPME C18 96-Pin Devices is performed as required to activate the adsorbent
- Coating is not allowed to dry between conditioning and extraction and between extraction and washing 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 C18 96-Pin Device methodology 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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