Supel™ BioSPME 96-Pin Devices

For Plasma Protein Binding Determination and Free Fraction Analyses

Supelco®
Analytical Products

Supel™ BioSPME 96-Pin Devices were developed to target the stringent specifications needed by bioanalytical laboratories, yet greatly improve upon the speed and simplicity compared with current sample preparation techniques employed for plasma protein binding studies today.

Primary Benefits of Supel™ BioSPME Devices

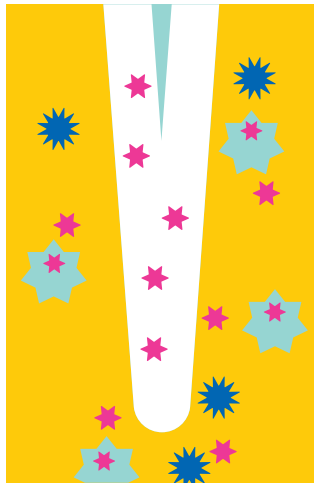
- >3x throughput and time savings compared to rapid equilibrium dialysis workflows
- Completely removes phospholipids, unlike rapid equilibrium dialysis methods
- Simple workflow can be fully automated or performed manually
- Accuracy and precision comparable to equilibrium dialysis and rapid equilibrium dialysis data
- Proven reproducibility across batches as well as across each 96-sample device

Protein Binding Determination for Drug Discovery

Protein binding properties of drugs are important to measure to understand the amount of free drug available in the blood. Historical methods to measure protein binding include equilibration of spiked plasma with drug-free buffer across a membrane, preventing the transfer of larger protein molecules into the buffer. This is an equilibrium dialysis method. Rapid equilibrium dialysis methods further reduce the equilibration time from the traditional 24 hours to 4 hours using specifically designed devices.

To improve upon current techniques utilized for protein binding determination in terms of workflow time and data quality, Supel™ BioSPME 96-Pin Devices were developed using Solid Phase Microextraction technology, similar in theory to the technology seen in SPME headspace analysis for GCMS workflows. These new devices have a 96-pin universal format that fit into the wells of standard 96-well plates and are compatible with high throughput automation. The tips of the pins are coated with a thin layer of adsorbent particles and can be directly lowered into the sample for extraction. The patented binder within the coating enables small analytes of interest to bind, while larger macromolecules cannot. This allows for a robust and selective non-exhaustive extraction process that can be used in both qualitative and quantitative applications, as shown in this brochure.

Supel™ BioSPME 96-Pin Devices measure the protein binding and reduce the sample preparation workflow from over 6 hours (using rapid equilibrium dialysis) to less than 2 hours. The Supel™ BioSPME method was applied to a range of compounds with molecular weights from 230-750 Da and a hydrophobicity or logP range from 1-5. Supel™ BioSPME can be utilized via automation robots (such as Hamilton® STARlet) or manual maneuvering, and the typical workflow is shown below in **Figure 1**. In the first step of the protocol, the pins are pre-conditioned with an organic solvent. The organic solvent is then washed off using a quick immersion into water prior to lowering the pins into a biological sample of choice. After extraction, another dip into water is done to remove any non-specifically adhering proteins prior to desorption into an organic solvent. A short and thin coating allows for a non-depletive extraction in most cases, using sample volumes as small as 100 µL.



Supel™ BioSPME pin plates are immersed directly into biological samples for small analytes of interest to be retained on the coated portion (red dots), while protein bound analytes (gray dots) are repelled.

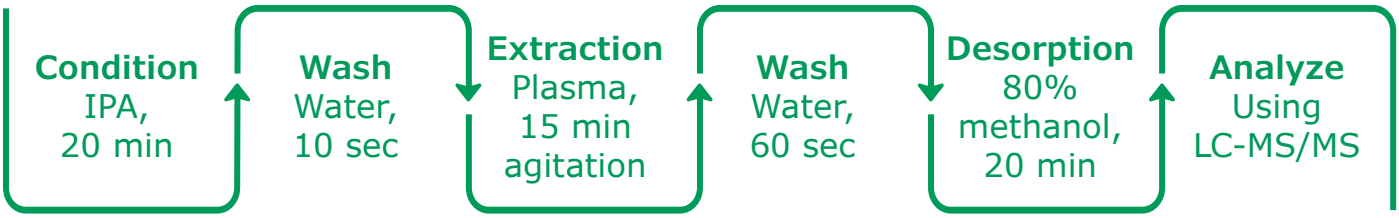


Figure 1 Overview of the steps for the Supel™ BioSPME workflow in the determination of free fraction of drug in plasma.

Comparison to Current Techniques (Rapid Equilibrium Dialysis)

Supel™ BioSPME 96-pin devices were directly compared with the rapid equilibrium dialysis technique, as the workflow of choice, as it is often considered the standard approach for plasma protein binding determination. Supel™ BioSPME 96-pin devices show numerous advantages over the rapid equilibrium dialysis technique, in terms of time savings, sample cleanliness, and simplified workflow, while still maintaining the same high standards of accuracy and reproducibility needed by the bioanalytical laboratories performing these workflows.

Results of Comparison

A comparison of the protein binding values between the rapid equilibrium dialysis method and Supel™ BioSPME method is shown below. Using the equations, **Eq. 5** and **Eq. 6** from the Deeper Dive Into How Protein Binding is Determined section below, the values in **Table 1** for analyte-protein bindings were determined for Supel™ BioSPME extractions. These values are in good agreement with values determined using rapid equilibrium dialysis devices and the reported literature values. These values are compared graphically in **Figure 2**.

Comparison of protein binding values between rapid equilibrium dialysis and Supel™ BioSPME methods

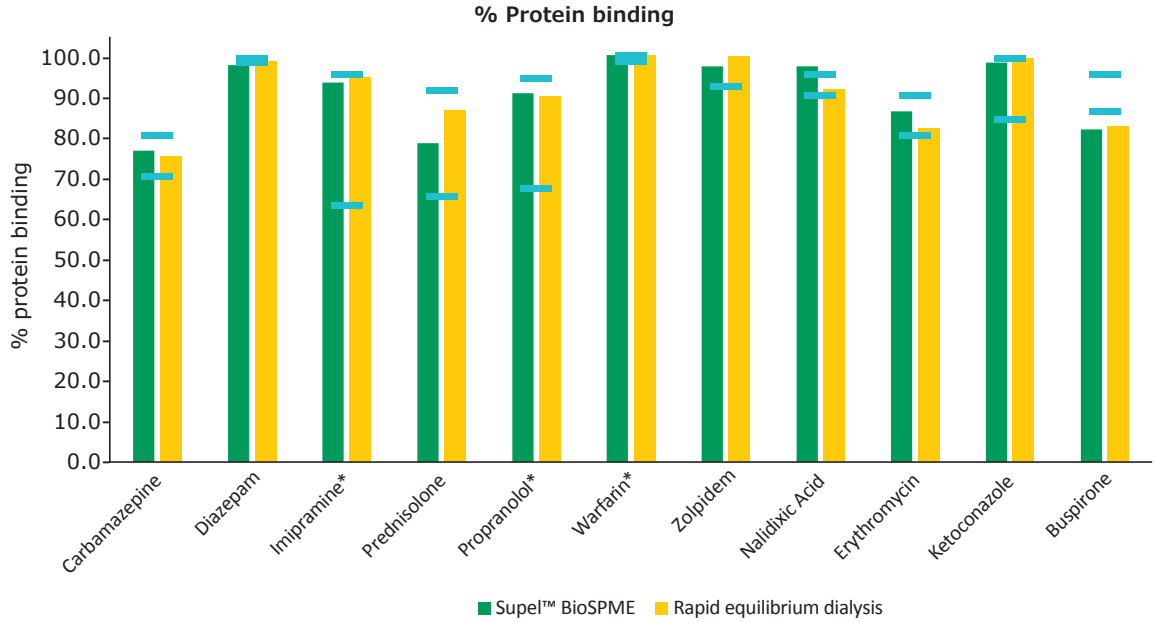


Figure 2. Comparison of protein binding values between Supel™ BioSPME and rapid equilibrium dialysis methods. The blue lines indicate the protein binding literature values range. Compounds with stars are charged at physiological pH.

Table 1. Binding values for the nine compounds from plasma using Supel™ BioSPME devices and 200 µL sample volumes (n=8).

Analyte	Concentration Spiked (ng/mL)	Supel BioSPME FB(%)	Rapid Equilibrium Dialysis FB (%)	Literature Values FB (%)
Carbamazepine	100	76.4	75.0	70-80%
Diazepam	100	97.3	98.2	98-99%
Imipramine*	100	92.6	94.4	63-95%
Prednisolone	100	78.2	86.1	65-91%
Propranolol*	100	90.5	89.7	67-94%
Warfarin*	2500	99.8	99.7	98.1-99.6%
Zolpidem	100	96.9	99.5	92%
Nalidixic Acid*	2000	97.0	91.4	90-95%
Erythromycin*	500	81.8	81.7	90%
Ketoconazole	500	96.8	99.0	84-99%
Buspirone*	100	81.6	82.3	86-95%

Fb = Fraction Bound

Comparison of Workflow Time: Supel™ BioSPME vs Rapid Equilibrium Dialysis

The time to perform each of the workflows was evaluated for comparison purposes as high throughput laboratories are always interested in optimizing efficiencies as much as possible. The Supel™ BioSPME workflow (<2 hours) takes one third of the amount of time as the rapid equilibrium dialysis workflow (6 hours). This results in the ability to increase throughput by 3 times to free up automation instrumentation and scientist time for other assays.

Table 2. Comparison of time requirement by the two methods

Rapid equilibrium dialysis Method	Step Time (min)	Supel™ BioSPME Method	Step Time (Min)
Prepare Samples	60	Prepare and Incubate Samples	60
Dialysis	240	Condition	20
Post sample preparation	40	Wash	0.2
Centrifugation	10	Extraction	15
Transfer for into vials for analysis	10	Wash	1
		Desorption	15
Total	**6 Hours**	**Total**	**<2 Hours**

Removal of Phospholipids (Matrix Effects)

Phospholipids are a problematic contaminant found in most biological samples, and it is oftentimes crucial to eliminate their presence in the sample to improve data quality. We compared the remaining levels of phospholipids present in sample after processing by either Supel™ BioSPME or rapid equilibrium dialysis, and the results are shown in **Table 3**. Since the Supel™ BioSPME devices do not retain phospholipids, it is natural for there to be complete phospholipid removal, whereas the rapid equilibrium dialysis workflow only removes around half of the phospholipids present in each sample. As a reminder, after dialysis using the rapid equilibrium dialysis method, protein precipitation is still required to be performed as clean plasma is added to the dialized buffer side to ensure the matrix is consistent between compartments, however, this does not remove most of the phospholipid interferences.

Table 3. Phospholipids remaining in sample by method

Method	# of samples	Average % Phospholipid Remaining	RSD
Supel™ BioSPME	5	<0.1	<0.01
Rapid equilibrium dialysis	5	56	7.8

Calculation to determine phospholipids remaining:

$$\%Phospholipid\ Remaining = \frac{\Sigma RED\ or\ BioSPME_{PPL}}{\Sigma ACN_{PPL}} \times 100\%$$

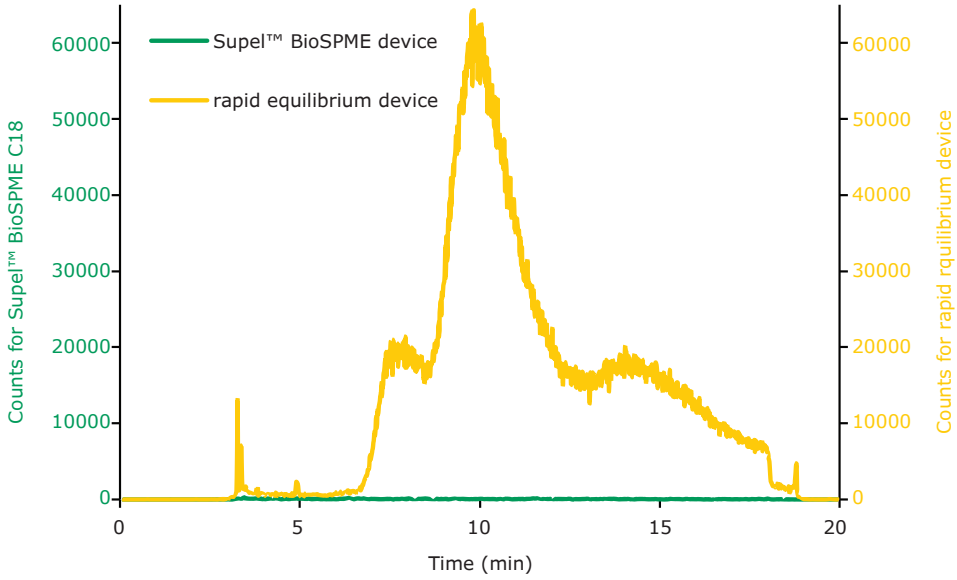


Figure 3. Phospholipid removal: Supel™ BioSPME device vs rapid equilibrium device

Accuracy and Precision

Accuracy of Supel™ BioSPME method in determining protein binding of a single compound across multiple lots of pin devices

It is highly recognized that lot-to-lot reproducibility is important to analytical laboratories that require ongoing consistent results. A comparison of 3 different batches within 3 different lots of Supel™ BioSPME 96-Pin Devices was performed to ensure consistent protein binding values over time.

To determine protein binding of carbamazepine, 8 spiked buffer and plasma pairs were tested on 6 different pin devices within each batch, as shown below. This was done for all 3 batches within 3 different lots of devices. As can be seen in **Figure 5**, same protein binding value was obtained for all batches and lots with 1-2% RSD. The average protein binding value observed across all 54 plates was 76.8 ± 2.2%, corresponding well with the 70-80% range of protein biding found in literature for carbamazepine.

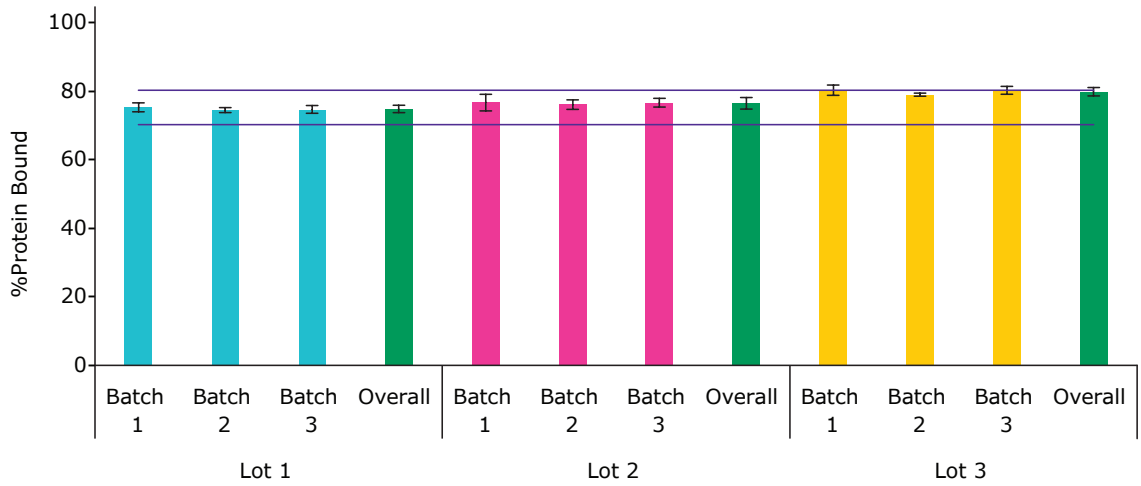


Figure 5. Protein binding values for carbamazepine obtained using 3 different lots of Supel™ BioSPME pin tools. Each batch represents six pin devices. The green bars represent the average across the 18 pin devices (or 3 batches) for each lot tested. Not shown is the overall average for the 54 pin tools (76.8 ± 2.2%). The lines represents the literature range for protein binding (70 – 80%).

Precision across a single pin device

In addition to showing consistency across multiple pin tools, an entire device was tested in what is termed the "8 + 88" sample setup. The eight represents one column for buffer extraction, and the 88 represents the remaining 11 columns for plasma extraction. The results across the device can be seen in **Figure 6** (analyte extraction). For the extraction of the analyte, the average extracted amount across 88 plasma samples was 7.31 ± 0.4 ng/mL (with RSD 4.8%). The average percent binding for the 88 samples was 75.9 ± 1.3%. As can be seen from **Figure 6** there was no bias across rows or columns of the 96-well plate.

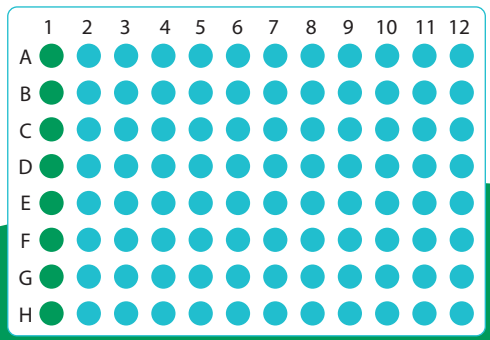


Figure 6. Overview of the "8 + 88 method". Column 1 (green) contains spiked buffer sample and columns 2 – 12 contains the spiked plasma (blue). The buffer at the beginning of each row was used to calculate the %protein binding across that row (ex. Buffer A1 was used to calculate the %protein binding associated with wells A2 - A12).

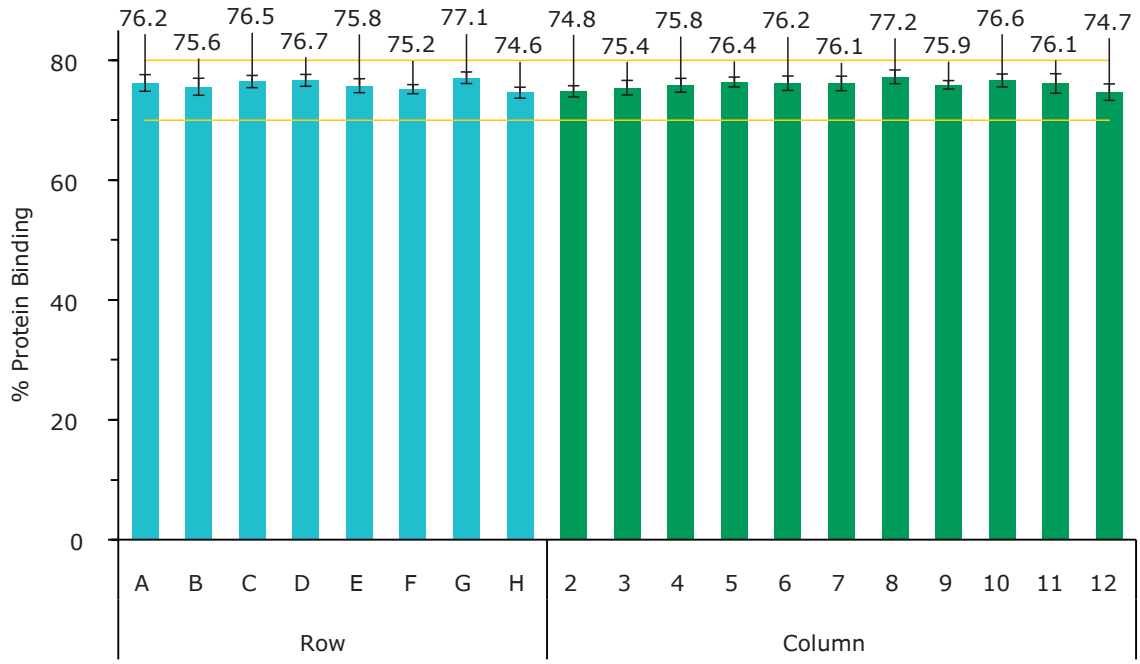


Figure 6 Percent bound with the standard deviation for each row and column.

Supel™ BioSPME technique is not only reproducible over time with batch-to-batch consistency, but also yields low %RSD in inter- and intra- device comparisons.

Summary

- The Supel™ BioSPME technique offers a 3x faster workflow for protein binding determination when compared with the rapid equilibrium dialysis method and is able to be performed via a fully automated robotic workflow
- Protein binding values determined by Supel™ BioSPME for the selected compounds with log P's ranging from 1 to 5 are all comparable to those prepared with the rapid equilibrium dialysis method, with the added benefit of cleaner samples through the removal of phospholipids
- Supel™ BioSPME offers peace of mind through batch-to-batch reproducibility and both inter- and intra-device consistency to match the high standards required by bioanalytical laboratories

Deeper Dive Into How Protein Binding is Determined

Determination of %Free Fraction (FU) by Supel™ BioSPME Method

The Supel™ BioSPME method determines the free concentration of analyte in plasma by comparing it with the extraction of the analyte from buffer samples, where 100% of the analyte is considered to be free of protein binding.

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

Eq. 1 $$\text{Free Fraction } (F_U) = \frac{\text{concentration free}}{\text{concentration total}} \times 100\%$$

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 the analyte. The amount extracted is independent of units and can be calculated using preferred quantities (e.g. nanograms or moles) Mfree, and extraction volume of plasma, Vplasma. 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 $$\text{concentration extracted from buffer, } B = \frac{M_{E,Buffer}}{V_{Buffer}}$$

M_E represents the mass extracted

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

Eq. 4 $$\text{Bound Fraction } (F_B) = 100\% - \text{Free Fraction } (F_U)$$

Eq. 5 $$\text{Bound Fraction } (F_B) = \frac{\text{concentration total} - \text{concentration free}}{\text{concentration total}} \times 100\%$$

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

Eq. 7 $$\text{Free fraction } (F_U) = \frac{P}{B} \times 100\%$$

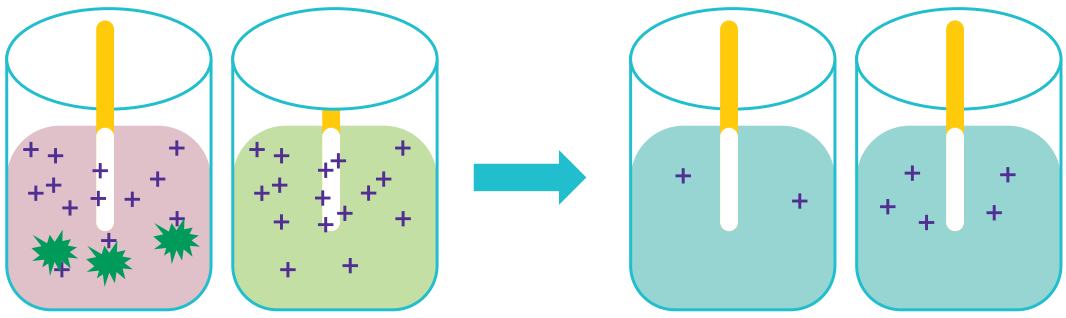


Figure 4. Representation of the extraction step (left) removing free analytes from plasma (pink) and buffer (green) and the analytes releasing into the desorption solution (right). The amount extracted does not greatly impact the concentration of free analyte which is termed non-depletive. As the buffer solution is considered 100% free, Supel™ BioSPME will extract more from buffer than from the plasma.

In cases where depletion of compounds from plasma was pronounced upon Supel™ BioSPME extraction (extraction exceeded 5% of total spiked analyte), a correction to the calculated Bound Fraction was 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. B^0 represents the concentration at which the samples were spiked originally. **Eq. 8** accounts for the concentration in solution after extraction on the fiber; the depletion of the analyte from sample.[6] **Eq. 6** and **Eq. 7**, do not take this factor into consideration. However, they provide accurate values when the extracted amount is less than 5%.

The percent depletion of plasma is shown in **Eq. 9**. P^0 represents the initial concentration of the analyte in the sample and P represents the amount extracted from the sample. In cases where the volume of the desorption ($V_{desorption}$) is equal to the volume of the sample for extraction (V_{sample}) **Eq. 9**, simplifies to **Eq. 10**.

Eq. 9 $$\text{Percent Plasma Depletion} = \frac{V_{desorption} \times P_{ave}}{V_{sample} \times P^0} \times 100\%$$

Eq. 10 $$\text{Percent Plasma Depletion} = \frac{P_{ave}}{P^0} \times 100\%$$

P^0 represents the initial concentration of the analyte in the sample

P_{ave} represents the average amount extracted from the sample

It is in important to be mindful of depletion as it can disturb the equilibrium between the analyte(s) of interest and the matrix components (i.e. proteins).

Quick Recap on Using Equations for the Comparison of Rapid Equilibrium Dialysis Versus Supel™ BioSPME Technique

Using the equations, **Eq. 5** and **Eq. 6**, the values in **Table 1** for analyte-protein bindings were determined from Supel™ BioSPME extractions. These values are in good agreement with values determined using rapid equilibrium dialysis devices and the reported literature values. These values are compared graphically in **Figure 2**.

Products and Methods

Both Automation and Manual Methods are available for download at **SigmaAldrich.com/biospme** with a full list of recommended products and accessories to successfully run your Supel™ BioSPME workflow.

Product List

Description	Cat. No.
Supel™ BioSPME C18 96-Pin Devices, 1 pack	**59680-U**
Supel™ BioSPME C18 96-Pin Devices, 10 pack	**59683-U**
Positioning Adapter (for Automation of Supel™ BioSPME), 1 pack	**59686-U**

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