Rapid Method Development for Bioanalysis with SPEC 96-well Method Development Kit

Application Note

BioPharma

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Introduction

LC analysis of biological samples (serum, plasma) with triple quadruple MS detection systems offers a plethora of data over a wide range of analyte concentrations. Furthermore, automation and data management systems have enabled researchers to increase productivity to the point where the rate limiting step in this type of analysis is typically sample preparation. While protein precipitation and dilute and shoot are rapid and easy, sample preparation by these nonspecific methods is often inadequate, especially for extremely low analyte concentrations, thereby necessitating a more specific and complicated approach. In contrast, solid phase extraction (SPE) is widely recognized as one of the most selective methods of sample preparation. However, despite its improved selectivity, SPE method development can be a challenging and time-consuming task. This application note describes a simple and rapid approach to developing new SPE methods using the SPEC 96-well method development plate. Solid Phase Extraction Concentrator (SPEC) is a monolithic-like extraction disk with a small void volume (relative to packed bed formats), delivering rapid flow and lower elution volumes.

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Materials and reagents

HPLC grade water, methanol (CH_3OH) and acetonitrile (CH_3CN), reagent grade formic acid, ammonium hydroxide and ammonium acetate, and SPEC Method Development Plate (p/n A59630).

Sample preparation

The Agilent SPEC 96-well method development plate includes 12 wells (by row) of each of the following phases: C2, C8, C18, C18 AR (acid resistant), CN, PH, MP1 (mixed mode C8 +SCX), and MP3 (similar to MP1, offering additional polar retention). Through the use of different chemistries for extraction, the variety of phases on the plate will provide an array of results, enabling the user to quickly and easily identify the phase and conditions that provide the optimum results.

1. Prepare solutions as shown in Table 1.
2. Prepare buffers for sample dilution (200 µL buffers for 100 µL plasma sample).

   Select an appropriate buffer that will maintain a neutral charge state for the analytes (pH above pKa for bases, below pKa for acids). For analytes with unknown pKas, use an ammonia buffer at approximately pH 10–12 for bases, formic acid buffer at approximately pH 2–3 for acids and acetate buffer at approximately pH 6–8 for neutral analytes. Analyte(s) must be basic (capable of bearing a positive charge) for MP1 and MP3. Use and references to these mixed mode phases can be ignored for neutral or acidic compounds. Prepare sufficient quantities for samples, conditioning and rinse steps. (10 samples plus one blank for the hydrophobic and CN phases and two samples plus two blanks for the mixed mode, conditioning and rinse steps at 2 × 200 µL for each well used).
3. Prepare 300 µL aliquot of analytes in plasma and buffer + optional internal standard (IS) (62 + 14 blanks).
4. For MP1 and MP3, dilute 2 additional samples in 2% formic acid solution.
5. Slowly and under very low vacuum (< 2 in Hg), condition wells 1–11 of the hydrophobic and CN phases, and well 1 of the MP1 and MP3 phases, with 200 µL followed by 200 µL sample dilution buffer. Condition well 2 of the MP1 and MP3 phases with 200 µL methanol, followed by 200 µL 2% formic acid buffer.
6. Load the samples onto the plate under low vacuum (2 in Hg) with the formic acid diluted MP1 and MP3 samples in well 2 of the corresponding row.
7. Rinse all wells with 200 µL of the sample dilution buffer (including column 1 of MP1 and MP3). Rinse well 2 of MP1 and MP3 with 200 µL 2% formic acid buffer.
8. Rinse well 1 of MP1 and MP3 with 200 µL 2% formic acid buffer.
9. Rinse well 1 and 2 of MP1 and MP3 with formic acid acidified methanol.
10. At this stage, when rinses are complete, apply high vacuum (> 10 in Hg) to all wells for 15 seconds.
11. Slowly, under moderate vacuum (2 to 5 in Hg), elute and collect the hydrophobic phases with 200 µL of methanol and acetonitrile solutions as shown in Figure 1. Elute with a second 200 µL aliquot into the same collection plate, combining elution aliquots. Elute blanks (column 11 and 12) with 100% methanol or acetonitrile (Figure 1).
12. Simultaneous with step 11, in 2 aliquots, elute all MP1 and MP3 wells with basified methanol solution.
13. Under a stream of heated nitrogen, evaporate all eluents to dryness and resuspend in appropriate buffer and volume for LC analysis. Analyze all samples compared to standard.

Table 1. Solutions used in the Analysis of Plasma using Agilent SPEC Method Development Plate

Solution	Concentration
Methanol	10, 30, 50, 70, and 90% in water (v/v)
Acetonitrile	10, 30, 50, 70, and 90% in water (v/v)
Formic acid	2% in water (v/v) 2% in methanol
Ammonium hydroxide	5% in methanol

	1	2	3	4	5	6	7	8	9	10	11	12
	10%		30%		50%		70%		90%		Blank	
	CH_3OH	CH_3CN	CH_3OH	CH_3CN	CH_3OH	CH_3CN	CH_3OH	CH_3CN	CH_3OH	CH_3CN	CH_3OH	CH_3CN
C18AR	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C18	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
PH	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C8	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
CN	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C2	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
MP1	Neutral sample	Charged sample	Not used									
MP3	Neutral sample	Charged sample										

Figure 1. 96-well plate layout and elution conditions when using Agilent SPEC method development kit.

Results and Discussion

Hydrophobic phases

Retention of analytes by the hydrophobic phases is based on van der Waals/hydrophobic interactions. In addition to a weak hydrophobic interaction, the cyanopropyl phase also retains analytes through a polar, dipole-dipole mechanism. Increased organic strength disrupts analyte-sorbent interactions and elutes the analyte when the eluent's affinity has exceeded that of the sorbent phase.

By plotting the peak area (concentration) versus percent organic, a profile of elution can be seen. Typically, at low organic levels, little or no analyte is seen. As organic concentration increases, some elution may be seen, followed by a precipitous rise in concentration, eventually reaching a plateau as most or all analyte is eluted. Figure 2 shows a typical elution profile. Depending on extent of hydrophobic and polar interactions, little to no retention (breakthrough at loading) may be seen with the CN phase.

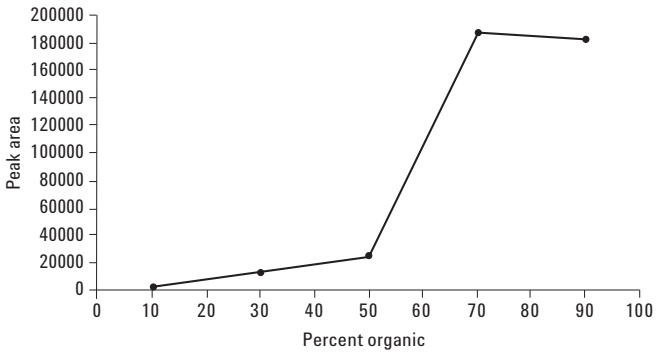

Figure 2. Typical elution profile obtained using the Agilent SPEC method development kit.

Based on the data in Figure 2, you would select a method with a 40–50% rinse followed by a 70% elution.

Mixed mode phases

Retention with MP1 and MP3 is based on hydrophobic and ion exchange interactions. In column 1, the initial retention mechanism is purely hydrophobic, based on the neutral state of the molecule. The aqueous rinse washes polar interferences from the sorbent. The addition of formic acid charges the analytes, thereby switching modes to ion-exchange, allowing a 100% organic rinse, eluting uncharged interferences.

In column 2, both the hydrophobic and ion-exchange mechanisms are used simultaneously because some compounds are insufficiently retained by only the hydrophobic mode. Simultaneous use of both modes may result in increased signal-to-noise. Analytes are eluted from the sorbent by simultaneous disruption of the hydrophobic interaction with methanol and neutralization with high pH.

Conclusions

The simple procedure described in this application note generates a significant volume of data in relatively little time. The six different hydrophobic phases of the SPEC method development plate will exhibit, in most cases, noticeable performance and response differences when subjected to the multiple conditions outlined. Visual inspection of the elution profiles and calculation of recoveries will let you determine the optimum SPE wash and elution conditions, noting that elution with lower concentrations of organic can often lead to cleaner extracts without sacrificing recoveries. The mixed mode phases on the same plate offer a convenient alternative method for comparison. Replicate plates or modification of conditions can be used to confirm and optimize an SPE method.

For More Information

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