

Technical Report

Systematic SPE Method Development

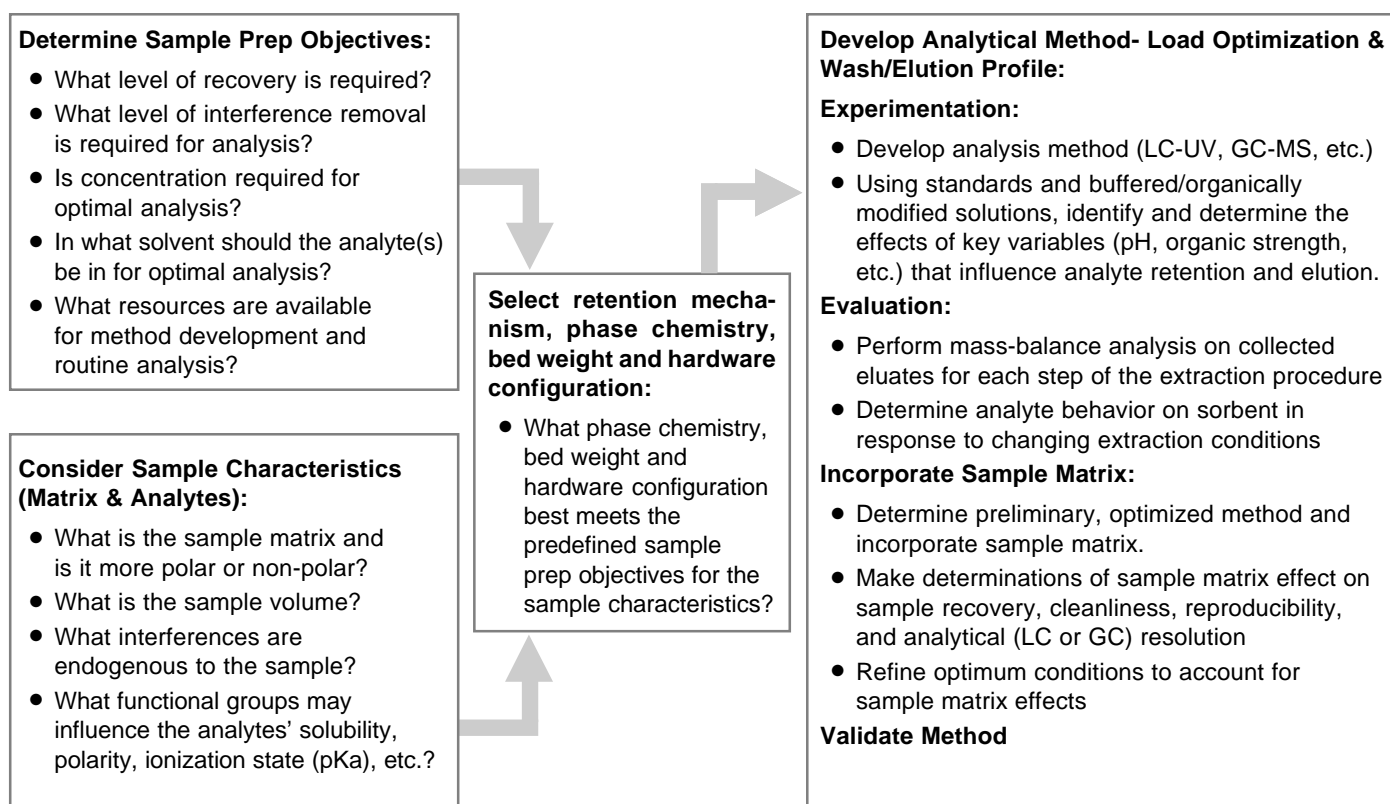
Comparison of a systematically developed method using Discovery DSC-8 SPE 96-Well Plate vs. a generic method using conventional C18 for the extraction and HPLC analysis of diazepam and its three major metabolites from goat serum

Authors: An Trinh, Product Manager, Liquid Separations, Dave Bell, HPLC Applications Group, Supelco, Bellefonte, PA

C18 has become the most commonly used phase chemistry for reversed-phase SPE due to its broad selectivity. However, there can be disadvantages to using C18 for some applications. Its higher hydrophobicity can lead to over retention of the analytes potentially leading to poor recovery/reproducibility from incomplete elution. For such applications, elution typically requires the use of stronger and/or larger volumes of solvent. The final eluate must then be evaporated and reconstituted with a solution suitable for LC-resolution and analysis. This prolongs and adds additional steps to the extraction procedure.

DSC-8 contains a monomerically bonded octyl chain with approximately half the carbon content of most C18 phases. Its less retentive nature allows for the rapid release of hydrophobic molecules using weaker eluants. Using the SPE method development approach illustrated in this report, a simple and highly selective extraction method using Discovery DSC-8 SPE 96-Well Plates was developed to recover diazepam and its three major metabolites from goat serum. When compared to a generic method using a conventional C18 phase, the systematic SPE method development approach provided a simpler method eliminating the final SPE eluate evaporation and reconstitution steps typical of most reversed-phase SPE procedures. Recoveries for the four compounds ranged from 90.0-99.9%, and RSDs were less than 3.5% for the 0.5µg/mL spike level tested.

SPE Method Development Process Overview



Determine Sample Prep Objectives

The objective of this study was to develop a simple extraction protocol that reproducibly achieves $\geq 90\%$ recovery for diazepam and its three major metabolites (temazepam, oxazepam, and desmethyl diazepam) from goat serum for HPLC-UV quantitation. Endogenous serum interferences should be substantially removed to simplify subsequent HPLC resolution, reduce analytical runtime ($\leq 8\text{min.}$), prolong HPLC column life, minimize misleading background responses, and achieve detection/quantitation limits of $\leq 0.5\mu\text{g/mL}$ serum. To minimize processing time, the final SPE eluate should be ready for direct injection/analysis. In other words, there should be no evaporation and reconstitution of the final eluate prior to LC analysis.

Consider the Sample Matrix

The sample matrix used in this study consists of 0.5mL samples of goat serum. Goat serum is an aqueous portion of blood from which platelets, corpuscles, and clotting factors (fibrinogen) have been removed. Endogenous interferences that should be considered include proteins such as albumin and globulins, lipids, salts, and carbohydrates.

Because of the aqueous nature of the sample matrix, reversed-phase or ion-exchange are potential retention mechanism choices.

Consider the Analyses of Interest

Diazepam (valium) is an antianxiety agent, anticonvulsant, and muscle relaxant used to treat mild to moderate anxiety, alcohol withdraw, epilepsy, and muscle spasms. It is adsorbed through the gastrointestinal tract and metabolizes to three major metabolites: temazepam, oxazepam, and desmethyl diazepam (Fig. 1). These compounds contain a benzodiazepine nucleus ideal for hydrophobic interaction under the reversed-phase retention mechanism (Fig. 2). The pKa for diazepam is 3.4 and ranges from 1.7-11.6 for the remaining benzodiazepine analogs. At different pH levels, various ionization states are induced for the different functional groups changing the relative selectivity for each of the compounds on a given sorbent.

Figure 1: Structures of Diazepam and Its Metabolites

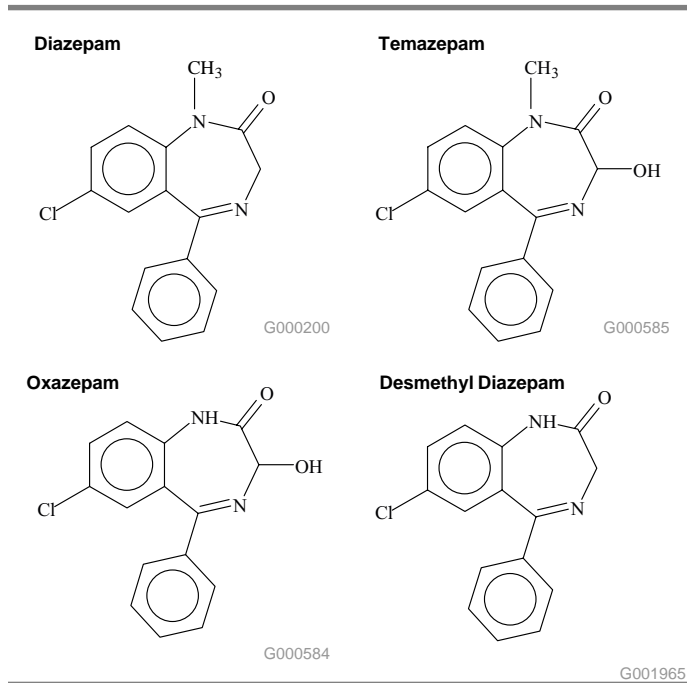
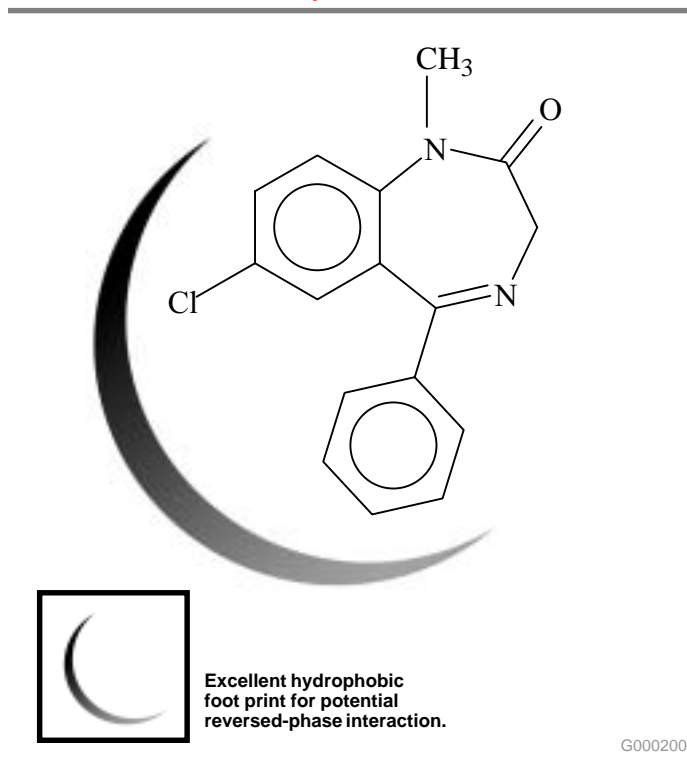


Figure 2: Diazepam Functional Groups that Influence Retention and Selectivity



Sample Prep Worksheet for Personal Use:

Determine Sample Prep Objectives:

What is your analytical technique (e.g., LC-UV, LC-MS, etc.)? _____

What is your optimal analytical run time (e.g., 2-5min.)? _____

What level of recovery is required to meet LOD/LOQ? _____

How do you plan to quantitate (against external standards and/or internal standards)? _____

What is your desired RSD (Inter- and Intra-day accuracy and precision)? _____

What level of interference removal is required for analysis? _____

What sample pretreatment steps may be required (dilution, clarification, pH adjustment, etc.)? _____

Is concentration required for optimal analysis? If yes, what is the desired elution and/or reconstitution volume? _____

In what solvent should the analyte(s) be in for optimal analysis? _____

What resources are available for method development and routine analysis? _____

Consider Sample Characteristics (matrix and analytes):

What is the sample matrix? _____

Is the sample matrix more polar or non-polar? _____

What is the sample volume? _____

What key interferences are endogenous to the sample? _____

What are the analyte(s) of interest? _____

What functional groups may influence the analytes' solubility, polarity, ionization states (pKa), etc.? _____

SPE Phase and Hardware Selection

Because the sample volume is 0.5mL, either a 1mL cartridge or a 96-well platform would be suitable for this application. The smaller bed weights (50-100mg for 1mL tubes; 25-100mg for 96-well) allow for small elution volumes. Smaller elution volumes provide higher analyte concentrations for increased sensitivity during analysis. Smaller elution volumes also allow for easier evaporation and buffer reconstitution prior to analysis.

The aqueous nature of the sample matrix and hydrophobic character of the analytes offer an excellent opportunity for reversed-phase retention. There are quite a number of reversed-phase sorbents available. Each of which offer varying degrees of hydrophobicity and selectivity that could be employed for this application. Because goat serum contains less interfering components relative to other mammalian species, weaker wash eluants may be employed. Also, because one of the original sample prep objectives asked for direct injection of the final SPE eluate, the final protocol will require weaker elution eluant (organically modified buffer) for final elution. In such situations, a less hydrophobic reversed-phase sorbent is recommended.

In this study, SPE method development was conducted on a Discovery DSC-8 SPE 96-Well Plate (100mg/well) (Fig. 3).

Figure 3: Supelco Discovery DSC-8 SPE 96-Well Plate and PlatePrep Vacuum Manifold



Experimentation

In SPE, selectivity is defined as the ability of the sorbent and extraction method to discriminate between the analyte(s) of interest and endogenous interferences within the sample matrix. In reversed-phase SPE, selectivity is typically governed by two main variables: pH and percent organic modifier.

By employing two or three experiments using standard solutions without the sample matrix, the researcher can systematically adjust these two variables. Through the use of standards, one can track the location of the analytes through the SPE process. By understanding how the analyte(s) interact with the sorbent under specific conditions, it allows for a systematic approach to finding the optimal sample prep conditions with greater efficiency and a higher degree of confidence.

Load Optimization

Standards containing 2.5µg/mL diazepam and its three major metabolites were prepared at neutral (25mM ammonium formate, pH 7.1) and basic pH (1% NH₄OH, pH 11). 1 mL of each of the standard test mixes were loaded on to the C8 wells previously conditioned and equilibrated with 1mL methanol and 1mL DI H₂O. The load eluate was collected and analyzed via HPLC-UV.

Note that although target detection level for the analysis was 0.5µg/mL serum, the load concentration was increased to 2.5µg/mL. This was to provide adequate signal response for detecting small percentages of analyte breakthrough. Also note that acidic load conditions were avoided due to the basic nature of the analytes of interest. The amine functional groups should be neutralized under basic conditions to provide optimal hydrophobic interaction between the analyte and sorbent alkyl chain functional groups.

Load Optimization Evaluation

A lack of analyte presence in the load eluate was found for both pH level conditions indicating adequate retention for both neutral and basic load conditions.

Since both pH levels provided adequate retention (no breakthrough) for all four of the analytes of interest, either pH levels could have been chosen for the load step. Neutral pH conditions were chosen for the load pH in this application.

Wash/Elution Profile

21 of the remaining C8 wells were conditioned and equilibrated with 1mL methanol and DI H₂O, and loaded with 1mL of the 2.5µg/mL neutral diazepam test mix. The samples were drawn through under the vacuum, and the respective wells were washed/eluted with 1mL of a test solvent consisting of 0, 5, 10, 20, 40, 60, 80, and 100% methanol in % NH₄OH, pH 11 (high pH), 25mM ammonium formate, pH 7.1 (neutral pH), and 10mM ammonium formate, pH 2.75 (low pH). The wash/elution eluate was collected and analyzed via HPLC-UV.

The purpose of this experiment was to systematically track the retention and elution of the analytes under various organic modifier and pH conditions.

Evaluation

A graphic representation was used to measure organic strength vs. peak area response for each of the pH conditions tested providing a wash/elution profile (Fig. 4).

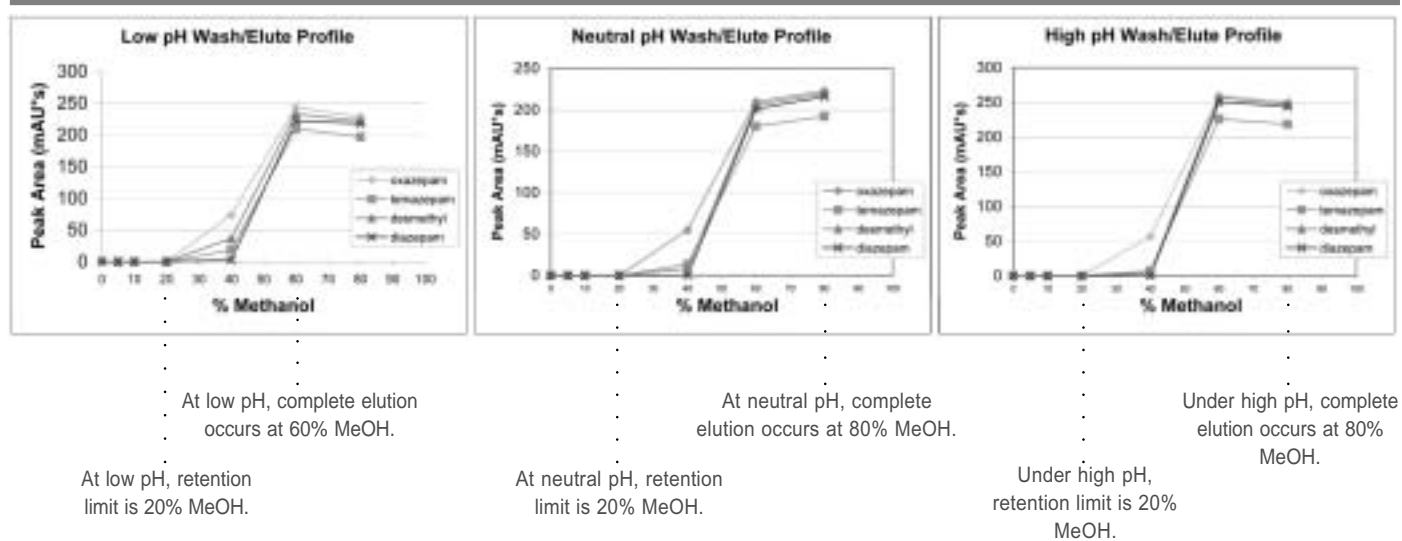
Diazepam is the most hydrophobic analyte of the four compounds withstanding elution at 40% methanol at each of the pH conditions tested. In contrast, oxazepam is the most polar compound eluting the most at 40% methanol at all three pH levels tested.

At low pH, the basic analytes were mostly in their ionic form. In this form, the benzodiazepines' amine functional groups coun-

teracted the hydrophobic interaction between the C8 alkyl chains and hydrophobic portion of the analytes. This allowed for easier and potentially more selective elution using weaker eluents.

Using the parameters suggested by the wash/elute profile, up to 20% methanol can be employed as a potential wash solvent. The wash elute profile suggested that under acidic conditions, the analytes of interest are weakest retained and full recovery can be achieved at 60% methanol. By eluting with a weaker eluent, direct analysis of the final eluent was possible without fear of poor resolution and peak shape.

Figure 4: Results of the Wash/Elution Profile for Diazepam and Its Three Major Metabolites on DSC-8



Incorporation of Sample Matrix and Comparison Against a Competitor Polymeric SPE Phase

By profiling the major parameters affecting analyte retention and elution, application specific guidelines have been established for defining, optimizing, and troubleshooting the extraction method. In this experiment, the goat serum sample matrix was incorporated into a method defined from data generated from the precursory load optimization and wash/elute profile studies (Table 1). For most applications, recovery values observed for real-matrix based solutions will parallel values obtained with standard solutions.

Table 1. Systematically Developed Method for Extracting Diazepam and its Major Metabolites from Goat Serum using Discovery DSC-8 SPE 96-Well Plate

SPE: Discovery DSC-8 SPE 96-Well Plate (100mg/well)

1. Condition and equilibrate each well with 1mL methanol and 1mL DI H₂O.
2. Load 1mL, 0.5µg/mL diazepam and metabolites spiked in goat serum diluted in 25mM ammonium formate, pH 7.1 (1:1, v/v); n=3.
3. Wash with 1mL 20% methanol in 25mM ammonium formate, pH 2.75.
4. Elute with 1mL 60% methanol in 25mM ammonium formate, pH 2.75.

The systematically developed DSC-8 SPE method was then compared against generic method using conventional C18 (Table 2).

Table 2. Generic Method for Extracting Diazepam and its Major Metabolites from Goat Serum using Conventional C18 Well Plate

SPE: Conventional C18 SPE 96-Well Plate (100mg/well)

1. Condition and equilibrate each well with 1mL methanol and 1mL DI H₂O.
2. Load 0.5µg/mL diazepam and metabolites spiked in goat serum diluted in 25mM ammonium formate, pH 7.1 (1:1, v/v); n=3.
3. Wash with 1mL 5% methanol in DI H₂O.
4. Elute with 1mL methanol.
5. Evaporate eluate with nitrogen purge (30°C; ~15min.), and reconstitute in 200µL HPLC mobile phase.

Results and Discussion

Diazepam and its three major metabolites were extracted from goat serum using a Discovery DSC-8 SPE 96-well plate. The method employed was defined from results obtained from preliminary load optimization and wash/elute profile studies. The systematically developed method was compared against a generic method using a conventional C18 SPE well plate. The resulting eluates were analyzed via HPLC-UV, and absolute recoveries and relative standard deviations were calculated against external standards (mobile phase sample matrix) not subjected to sample preparation (Table 3).

Table 3. Efficiency of Absolute Recovery of 0.5µg/mL Diazepam and Metabolites on Systematically Developed Method Using DSC-8 vs. Generic Method on Conventional C18

Compound	%Recovery ± RSD (n=3)	
	Systematically Developed Method on Discovery DSC-8	Generic Method On Conventional C18
1. Oxazepam	94.7 ± 1.2%	82.8 ± 4.0%
2. Temazepam	99.9 ± 1.1%	89.1 ± 4.0%
3. Desmethyl diazepam	94.2 ± 1.8%	82.4 ± 5.0%
4. Diazepam	90.0 ± 3.4%	68.5 ± 9.1%

Simpler SPE Procedure, Clean Extracts, & Reduced Analytical Run Time

The systematically developed method using the DSC-8 well plate provided a simpler procedure than the generic method employed with the conventional C18 phase. Most reversed-phase SPE protocols using C18 require a 100% organic elution. Buffer is then added to the organic eluate, or the eluate must be evaporated and reconstituted with mobile phase prior to LC resolution. This adds an additional step and further prolongs the extraction procedure. Because the C18 protocol utilized a methanol elution, the final eluate required an evaporation and reconstitution step prior to LC analysis. On the DSC-8 phase, a weaker eluent (60% methanol in 25mM ammonium formate, pH 2.75) was employed allowing for direction injection/analysis of the final eluate. This reduced the number of overall steps and minimized the processing time for the extraction procedure.

The systematically developed DSC-8 method provided clean extracts signified by chromatograms with low background deficient of misleading peaks/responses (Fig. 5). The clean extracts allowed for minimal analytical run times (6 min.) resulting in faster more accurate results.

For expert answers to your questions, contact our Technical Service Department:

Phone 800-359-3041, 814-359-3041

Fax 800-359-3044, 814-359-5468

E-mail techservice@sial.com

To request Supelco's free technical literature, call 800-359-0682 or visit

sigma-aldrich.com/supelco-literature

sigma-aldrich.com/supelco

Order/Customer Service 800-247-6628, 800-325-3010 • Fax 800-325-5052 • E-mail supelco@sial.com

Technical Service 800-359-3041, 814-359-3041 • Fax 800-359-3044, 814-359-5468 • E-mail techservice@sial.com

SUPELCO • 595 North Harrison Road, Bellefonte, PA 16823-0048 • 814-359-3441



We are committed to the success of our Customers, Employees and Shareholders through leadership in Life Science, High Technology and Service.

The SIGMA-ALDRICH Family  SIGMA  ALDRICH  Fluka  Riedel-deHaen  SUPELCO

© 2003 Sigma-Aldrich Co. Printed in USA. Supelco brand products are sold through Sigma-Aldrich, Inc. Sigma-Aldrich, Inc. warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see reverse side of the invoice or packing slip.



T403039
FOP