

# Optimisation of solid phase extraction for the analysis of benzodiazepines from plasma

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## Introduction

Benzodiazepines possess sedative, hypnotic, anxiolytic, anticonvulsant, muscle relaxant, and amnesic actions, which are useful in a variety of indications such as alcohol dependence, seizures, anxiety, panic, agitation and insomnia. This class of compounds has also been associated with drug abuse.

The analysis of benzodiazepines within biological systems allows effective treatment of a range of anxiety based conditions. This requires a combination of mass spectrometry, chromatography and also sample preparation to allow the quantitation of these compounds within the biological fluid, typically plasma. The sample preparation has a significant effect on the quality of the data being produced. The subsequent sections detail the investigation into various sample preparation techniques for the removal of endogenous matrix components from the original plasma sample.

## Methods

### Approaches

Two sample preparation techniques were investigated with the aim of determining the matrix components left after the extraction process:

#### 1. Protein Precipitation

100 µl of plasma was added to 300 µl of ACN. Supernatant blown down and reconstituted in 500µL of water-acetonitrile-formic acid (95:5:0.1 v/v)

#### 2. Solid Phase extraction

Details of the optimisation procedure are given below using either the HyperSEP Retain PEP, or the HyperSEP Retain CX solid phase extraction sorbents. Optimisation of the washes is given in the subsequent section

### Chromatographic and Detection Methods:

Samples were prepared using the two different sample preparation techniques and then analysed using LC-MS.

Instrumentation: Thermo Scientific Accela UHPLC with Thermo Scientific MSQ mass spectrometer.

Column – Hypersil GOLD 1.9µm, 50 x 2.1 mm

Mobile Phase A – 0.1% Formic Acid (aq); Mobile Phase B – 0.1% Formic Acid in ACN.

Gradient: 95% [A] and 5% [B] for 0.5 min, linear change to 5% [A] and 95% [B] in 3 min, then change to 95% [A] and 5% [B] and hold for 1 min.

Flow rate: 0.6mL/min; Injection volume: 10µL

Detection: positive ESI; for elution profile, 12 benzodiazepines SIM for each [M+H]<sup>+</sup>; for blank plasma extracts, full scan from m/z 100 - 1000

### Elution Profiles

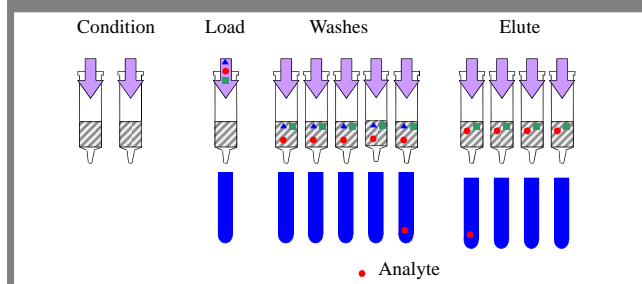
This offers a quick optimisation of wash and elution conditions and recovery.

Compounds are loaded onto a single SPE cartridge and washed with increasing elutropic solvent strength (Table 1). Flow injection analysis of the eluents will determine the optimal extraction conditions, Figure 1.

Table 1. SPE methodology

	HyperSEP Retain PEP	HyperSEP Retain CX
Bed weight	30mg/mL	30mg/mL
Conditioning	1mL MeOH 1mL water	1mL 0.1% formic acid in methanol 1mL 0.1% formic acid in water
Load	1mL 500ng/mL sample in water	1mL 500ng/mL sample in 0.1% formic acid in water
Wash	1mL MeOH (10%-90% tested)	1mL 0.1% formic acid in water 1mL 0.1% formic acid in methanol 1mL 5% ammonia in MeOH (10%-90% tested)
Elute	4 x 0.5mL 100% methanol	4 x 0.5mL 5% ammonia in 100% MeOH

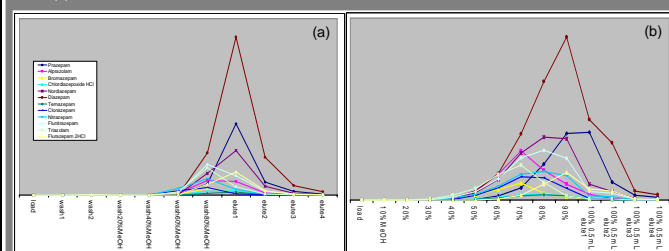
Figure 1. Overview of the elution profile for optimisation of the SPE method



## Results

Figure 2 shows the experimental data for the optimisation of the benzodiazepines extraction on the HyperSEP Retain CX and HyperSEP Retain PEP materials. From this graph the optimum wash and elution conditions for all of the compounds can be readily determined.

FIGURE 2. Elution profile for 12 benzodiazepines on HyperSEP Retain CX (a) and HyperSEP Retain PEP (b).



## Matrix components after protein precipitation

Analysis of the matrix was achieved using TIC chromatograms. Since the chromatography was not optimised for the matrix components, several aqueous blank injections cycles were also performed subsequent to the plasma extract injection to elute highly retained components. This is important information as it is generally assumed that the column has not altered from one injection to the next.

It can be seen from Figure 3 that there are substantial amounts of matrix components still remaining in the sample after protein precipitation, in particular there are some very defined regions, of early eluting polar compounds, and then more hydrophobic compounds which elute at later retention times.

FIGURE 3. Comparison of clean system (a) and a TIC chromatogram obtained using a protein precipitated plasma sample (b).

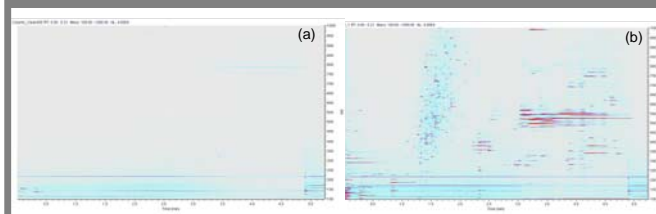
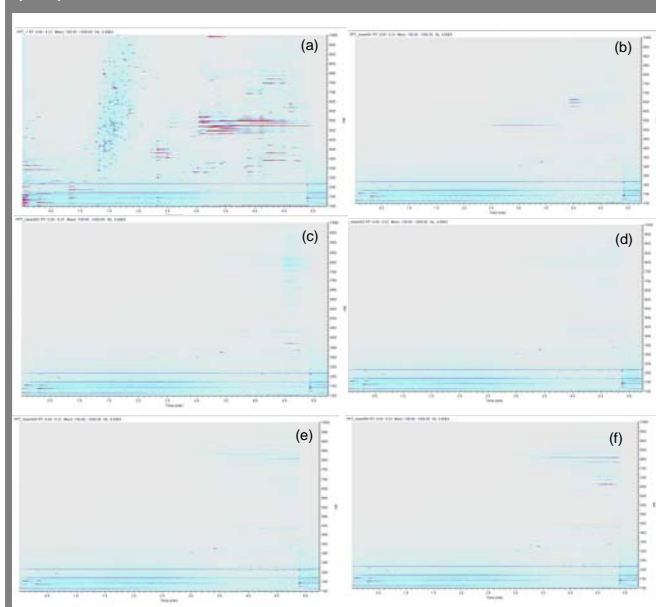


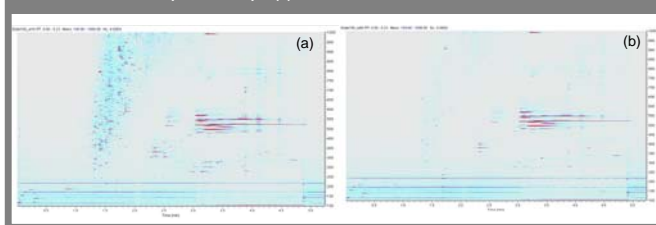
FIGURE 4. Protein precipitation sample (a) followed by a series of aqueous blank injections (b to f).



## Optimising the wash conditions

The use of generic methods in SPE with a 5 or 10% organic wash results in a substantial amount of matrix components still remaining in the injected samples. Increasing the wash concentration to 60% methanol removed the majority of the polar compounds but did not remove the hydrophobic components, which required up to 80 or 90% methanol washing before the extract became clean.

FIGURE 5. Comparison of TIC chromatogram obtained after SPE with 10% methanol wash (a) and 60% methanol wash of plasma sample (b).



## Conclusions

The importance of the sample preparation technique in removing endogenous matrix components has been demonstrated. It has also been shown that with typical methods used in routine bioanalysis it takes several injection cycles to remove the endogenous matrix components from the system. This suggests that the chromatographic system changes on the first few injections before stabilising.

A quick approach to the optimisation of solid phase extraction method has been presented with two proposed optimised methods for the determination of a series of twelve benzodiazepines.

For additional information, please visit our Chromatography Resource Centre which can be found at:

[www.thermoscientific.com/chromatography](http://www.thermoscientific.com/chromatography)

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