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Generic SPE protocol for peptide clean-up and concentration

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Goal

Description of a generic, reproducible and robust procedure that can be used to clean up and concentrate peptide samples. The procedure can remove unwanted buffer, reagents, and any physical particulates from the sample while maintaining high levels of analyte recovery and reproducibility. An additional benefit is the ability to concentrate the sample prior to analysis.

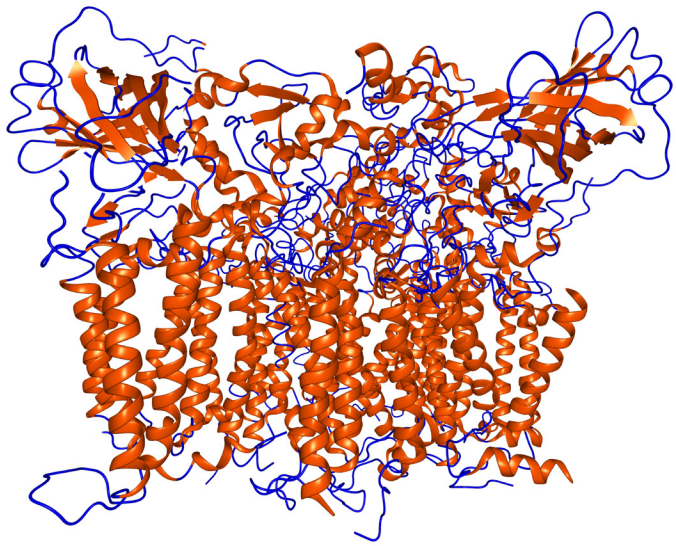
The method is performed using micro-elution solid phase extraction (SPE) and, therefore, benefits from removal of post-extraction sample evaporation and reconstitution required by traditional scale SPE. Removal of these steps eliminates issues with solubility and non-specific binding (NSB) that are often associated with peptide analysis.

Introduction

Peptide analysis can present a number of issues that affect detector response, due to the presence of unwanted interferences from either the matrix or from reagents and other additives used to facilitate protein digestion. Sample preparation techniques employed to remove these interferences are required to be quick, simple, and generic. Reproducibility is also important as this enables users to confidently assign data differences to the sample and not the methodological conditions used.

Generic micro-elution SPE methods can be successfully employed for a nontargeted workflow, removing only the unwanted reagents and particulates from the digested sample while maintaining a high recovery and extraction reproducibility of the peptides. An additional concentration factor can be applied to assist with analysis.

The Thermo Scientific™ SOLAμ™ micro-elution SPE device is built on a polymeric backbone containing both reversed-phase and polar retentive moieties. SOLAμ SPE provides reproducibility, robustness, and ease of use at low elution volumes by utilizing the



revolutionary Thermo Scientific™ SOLA™ Solid Phase Extraction technology. This removes the need for frits by delivering a robust, reproducible format that ensures highly consistent results at low elution volumes providing;

- Lower sample failures due to high reproducibility at low elution volumes
- Increased sensitivity due to lower elution volumes
- The ability to process samples that are limited in volume
- Improved stability of bio-molecules by reduction of adsorption and solvation issues

Here we show a proof of concept for a generic clean-up protocol. Eight well-characterized peptides were processed and monitored for recovery and reproducibility. Four of the peptides were derived from digested cytochrome c. Four additional peptides were spiked into the digested sample before processing in order to broaden the range of peptides used for assessment of the protocol.

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Experimental

Sample preparation

- SOLAμ HRP 96 well plate
- Thermo Scientific™ SMART Digest™ kit with SOLAμ HRP

Chemicals

- Water
- Acetonitrile (ACN)
- Formic acid (FA)
- Trifluoroacetic acid (TFA)

Cytochrome c, leu-enkephalin, angiotensin 1, angiotensin 2, and neurotensin were purchased from reputable sources

- 96 well square well microplate

Separation

- Thermo Scientific™ Acclaim™ RSLC 120, C18, 2.2 µm analytical LC column (2.1 × 100 mm)

Sample handling equipment

- 96 well positive pressure manifold

Separation preparation

Additional exogenous peptides were added to digested cytochrome c (20 µg) samples. Both the cytochrome c derived and the exogenous peptides were processed and analyzed (Figure 1). Results were compared to reagent blanks that were processed and spiked with peptide standards post extract (Figure 2).

Analytical conditions

All samples were analyzed using the Thermo Scientific™ Vanquish™ UHPLC system. Separation on an Acclaim RSLC analytical column was achieved with a linear gradient of 0.1% formic acid in water to 0.1% formic acid in acetonitrile over 15 minutes.

Detection was performed in full scan mode on a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer, and the data was processed using Thermo Scientific™ Xcalibur™ Quant software version 3.0.63 with the mass to charge ratios (m/z) in Table 1.

Data analysis

Thermo Scientific™ Pepfinder™ 2.0 was used to analyze the resulting data set.

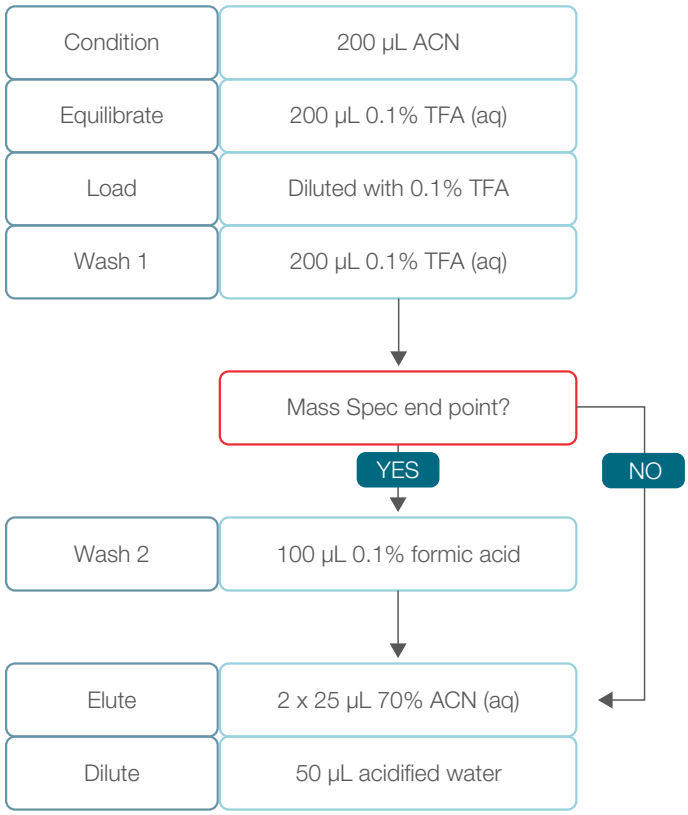
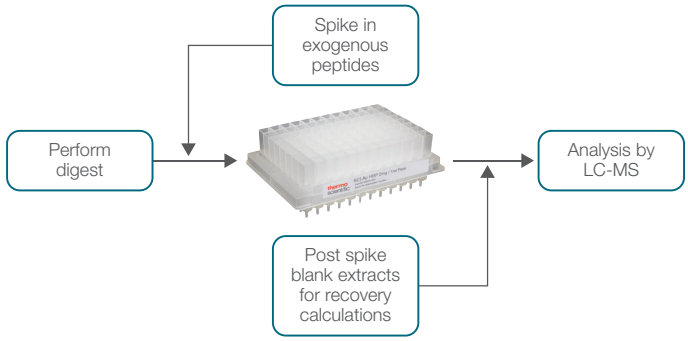


Figure 1. Clean-up workflow.



$$\text{Recovery} = \frac{\text{Average Response of Sample}}{\text{Average Response of Overspike}} \times 100$$

Figure 2. Recovery calculation method

Results and discussion

A total of eight peptides were analyzed for assessment of the method. Four well-characterized peptides derived from cytochrome c, along with four well-characterized exogenous peptides, were spiked in post digestion. Table 1 and Figure 3 show recovery levels of each peptide assessed along with the reproducibility (n=6). Recovery levels of 86% or higher were achieved in seven out of eight peptides with single digit %RSD on all peptides. This demonstrates a high level of reproducibility across the range of peptides assessed.

Table 1. Peptide recovery data

Sample	Amino Acid Sequence	MS *(m/z)*	Recovery (%)	Precision (%RSD)
Cytochrome c peptide	EDLIAYLK	483.27301	101%	4%
	GITWGEETLMEYLENPKK	711.33099	113%	5%
	MIFAGIK	779.44641	51%	9%
	TGPNLHGLFGR	390.21155	109%	6%
Leu_Enkephalin	YGGFL	556.27526	97%	3%
Angiotensin_I	DRVYIHPFHL	432.8987	93%	7%
Angiotensin_II	DRVYIHPF	523.77349	86%	7%
Neurotensin	ELYENKPRRPYIL	558.30907	91%	3%

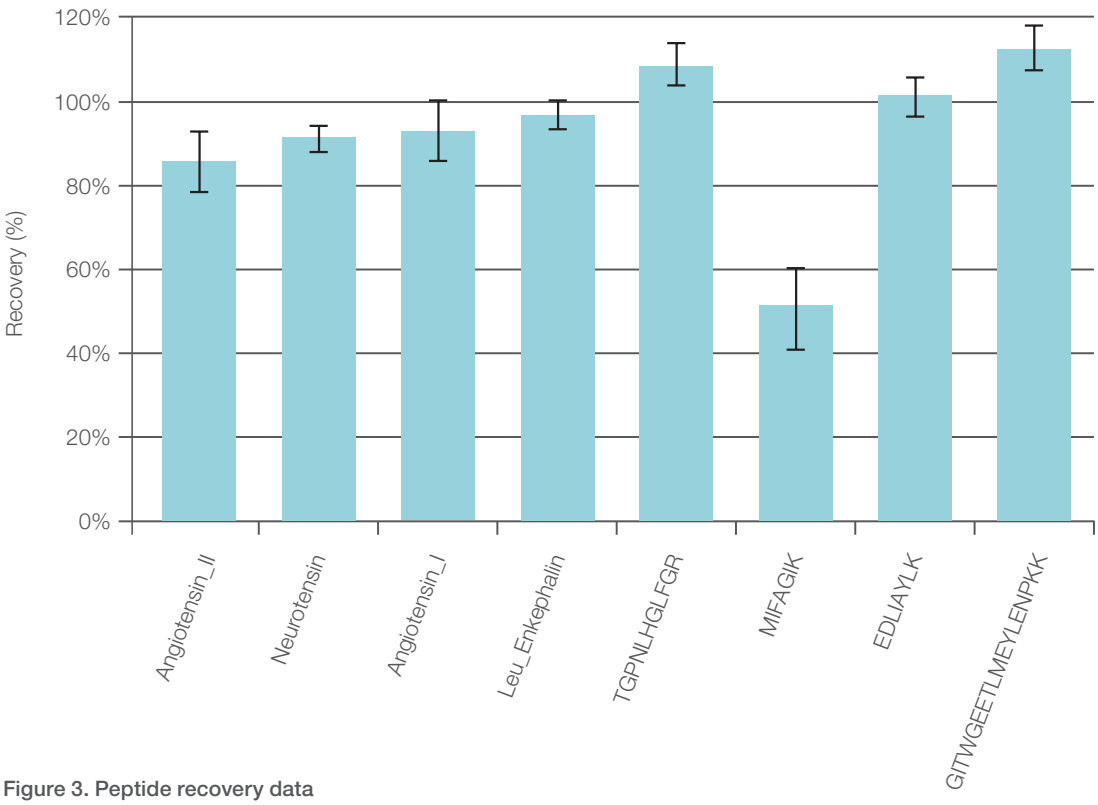


Figure 3. Peptide recovery data

The final extracts demonstrated 85% protein coverage. This was calculated using PepFinder software against the theoretical number of peptides possible from cytochrome c. The peptide coverage map is shown in Figure 4.

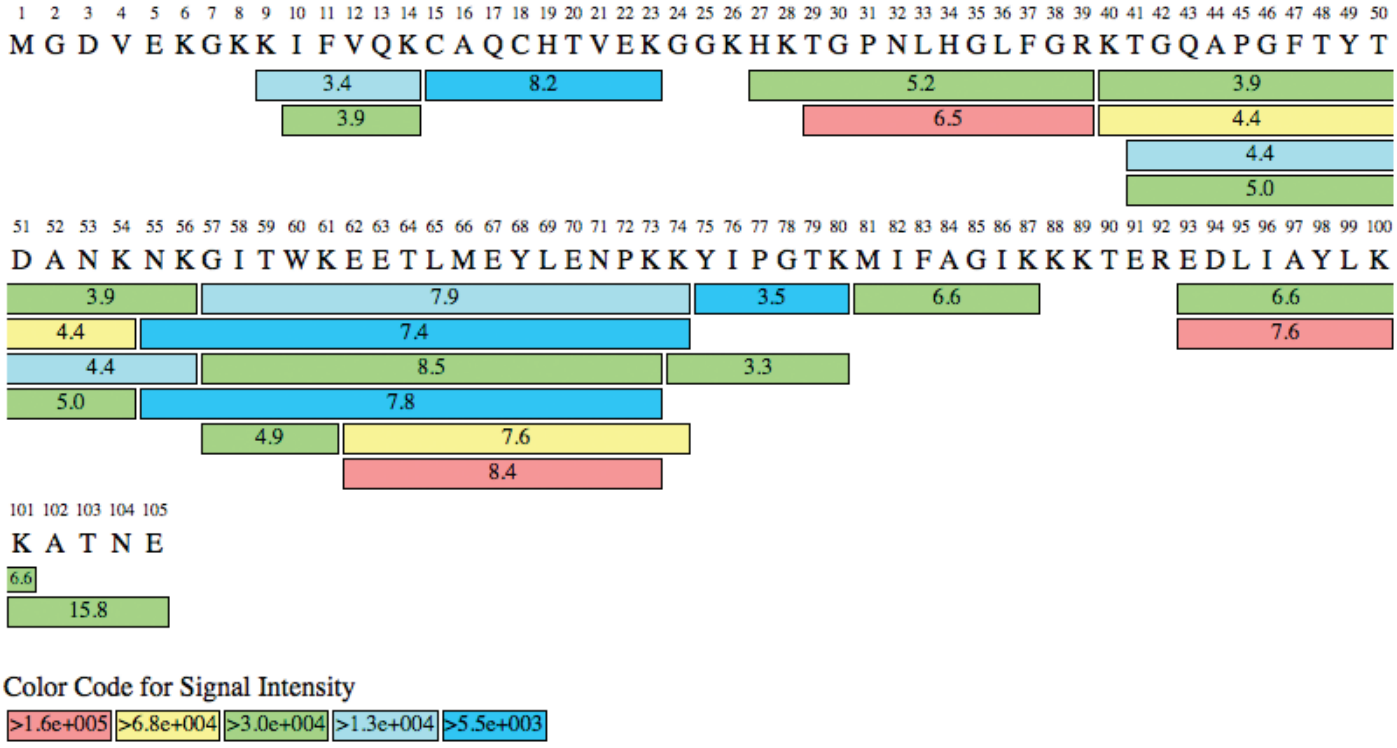


Figure 4. Peptide coverage data for cytochrome c
*Sequence of cytochrome c is shown. Each peptide is identified here by amino acid sequence, color coded for detection intensity. The chromatographic retention time is denoted by the number within each box (minutes).

Conclusion

- Evaluation of the generic clean-up procedure shows high levels of recovery and reproducibility of the peptides used for assessment. An increase in detector response can be achieved through sample concentration.
- Despite one low recovery value for MIFAGIK, levels of precision remained high throughout. Furthermore, an assessment of sequence coverage revealed that this protocol maintains a high overall percentage of peptides, demonstrating the use of this method as a nontargeted approach for peptide analysis.
- The described workflow both cleaned and concentrated the digest sample. Due to the format, up to 96 samples can be processed in as little as 15 minutes, making this workflow well suited to a high-throughput environment.

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