# Improvement in Speed and Reproducibility of Protein Digestion and Peptide Quantitation, Utilizing Novel Sample Preparation Technology in a Full Solution Workflow

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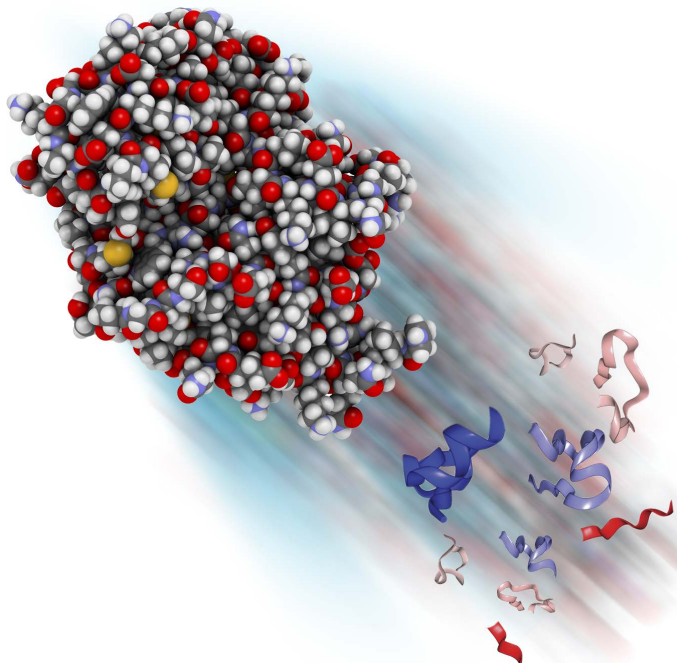
Application Note 21198

**Key Words**

SMART Digest kit, SOLAμ, Vanquish, peptides, protein, bottom up proteomics, proteomics, trypsin digestion, buffer, mAb, biomolecules, biotherapeutics, biopharmaceutical, biocompatible UHPLC, Acclaim C18 RSLC, peptide mapping, in-solution digestion, UHPLC, accurate mass, QE, Orbitrap, peptide quantitation, SPE, solid-phase extraction, Q Exactive Plus

## Goal

To describe a rapid and precise protocol for peptide analysis utilizing immobilized, heat stable trypsin and micro-elution solid-phase extraction (SPE) coupled with next generation ultra-high pressure liquid chromatography and high resolution mass spectrometry (UHPLC-HRMS). Traditional trypsin digest protocols are time consuming, with some protocols taking up to one and a half days to complete and involving multiple steps including evaporation and reconstitution. This adds potential for a high degree of variability. The Thermo Scientific™ SMART Digest™ kit was used to digest cytochrome C with a 10 minute digestion protocol. This was followed by Thermo Scientific™ SOLAμ™ SPE plates to clean and concentrate the digest prior to injection onto the Thermo Scientific™ Vanquish™ UHPLC system. Following separation on a Thermo Scientific™ Acclaim™ C18 RSLC analytical column, high-resolution mass spectrometry was performed using a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer. A high-throughput, reproducible workflow was achieved that can be applied to nontargeted, semitargeted, or targeted quantitative environment for peptide analysis.



## Introduction

A fundamental requirement of peptide mapping and quantitative analysis workflows is reproducibility. This enables users to confidently assign data differences to the sample and not the methodological conditions used.

The current approach for peptide mapping and quantitative analysis involves in-solution trypsin digestion of proteins overnight. This protocol is time consuming and requires a number of different steps, including protein assay, denaturation, alkylation, and reduction, which can differ between laboratories and make method transfer and data analysis between user groups problematic.

Additionally, due to the number of steps required, in-solution digestion protocols are highly laborious, increasing the potential for user error. As a result, in-solution digestion often leads to irreproducible protein cleavage which manifests in variation in the chromatographic profile. This prevents the adoption of robust, generic methodologies resulting in reduced throughput and return on investment.

The SMART Digest kit eliminates these issues by providing a protocol that is:

- Highly reproducible
- Quick and easy to use
- Detergent free
- Less prone to chemically-induced post translational modification (PTMs)
- Autolysis-free
- Highly amenable to automation

Prior to analyzing digested samples it is common to perform sample cleanup such as centrifugation, filtration, or solid-phase extraction (SPE). This removes unwanted chemicals (such as detergents) that can interfere with the downstream mass spectrometric detection.

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Centrifugation cannot always provide the level of cleanup required and exposes the detection system to unwanted levels of contaminants. Consequences can be physical, such as a blocked injection needle, as well as analytical, such as reduced column life or detection variability.

Filtration can be used to prevent the physical issues from occurring but offers little in the removal of excess buffers and reagents. Furthermore, the apparatus used in filtration can increase assay variability due to unpredictable binding of molecules to sample handling devices.

SPE provides a solution to both issues by filtering the digest while selectively removing the reagents. A generic method can be employed for a nontargeted workflow, removing only the unwanted reagents from the digest while maintaining a high recovery and reproducibility.

SOLAμ SPE plates provide 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 while delivering a robust, reproducible format which 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 which are limited in volume
- Improved stability of bio-molecules by reduction of adsorption and solvation issues

SOLAμ HRP is a micro-elution SPE device built on a polymeric backbone, containing both reversed-phase and polar-retentive moieties. In addition to providing high levels of recovery and reproducibility, it enables an additional concentration factor to be achieved without the need for evaporation and reconstitution. Removal of the evaporation step reduces non specific binding when compared to traditional-scale SPE where evaporation and reconstitution are required.

Here we compare two protocols of sample cleanup following protein digestion with the SMART digest kit: filtration and SOLAμ HRP SPE. A total of eight peptides were assessed, four from a cytochrome C digest (to assess the SMART digest) and four exogenous peptides spiked in post digest (to assess the reproducibility of the cleanup methods). Recovery and reproducibility of all eight peptides were measured, allowing an assessment of each stage of the workflow.

All samples were analyzed using the Vanquish UHPLC system, which is optimized to reduce extra column band dispersion and allow users to significantly improve the separation power of their analytical assays. The 1500 bar pressure capability of the Vanquish pump enables an extended range of flow rates to be employed allowing for faster separations and higher throughput. Separation on an Acclaim C18 RSLC analytical column was achieved within 15 minutes.

Detection was performed on a Q Exactive Plus Orbitrap MS, a benchtop LC-MS system designed for high-performance, high-throughput screening, compound identification, and quantitative analysis. With its Orbitrap™ mass analyzer, the Q Exactive Plus system delivers high-resolution, accurate-mass (HRAM) full-scan MS for fast, precise, and reproducible results with analytical confidence.

## Experimental

### Digestion

- SMART Digest kit (P/N 60109-101)

  SMART Digest with SOLAμ HRP kit (P/N 60109-103)

### Chemicals

- Fisher Scientific™ Optima™ water (P/N 10095164)
- Fisher Scientific Optima acetonitrile (ACN) (P/N 10001334)
- Thermo Scientific™ Pierce™ formic acid (FA) (P/N 10628654)
- Fisher Scientific™ trifluoroacetic acid (TFA) (P/N 10294110)

Cytochrome C, leu_enkephalin, angiotension 1, angiotensin 2, and neurotensin were purchased from reputable sources.

### Sample Handling Equipment

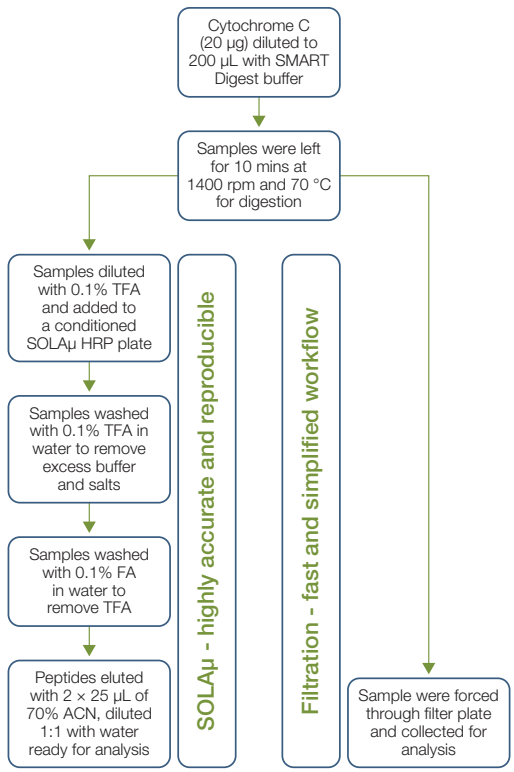
- 96 well square well microplate (P/N 60180-P202)
- 96 well positive pressure manifold (P/N 60103-357)

### Separation

- Acclaim RSLC 120, C18, 2.2 μm analytical column (2.1 × 100 mm) (P/N 068982)

It is also recommended that a heater/shaker equipped with PCR block be used to perform the digest.

### Sample Preparation



### Separation Conditions

| | |
|---|---|
| Instrumentation | Vanquish UHPLC system consisting of: |
| | Binary pump H (P/N VH-A10-A) |
| | Split sampler HT (P/N VH-A10-A) |
| | Column compartment H (P/N VH-C10-A) |
| | Active pre-heater (6732.0110) |
| | Post column cooler, 1 µL (6732.0510) |
| Mobile phase A | Water + 0.1% formic acid |
| Mobile phase B | Acetonitrile + 0.1% formic acid |
| Column temp. | 30 °C (Still Air) |
| Injection details | 2 µL |
| Gradient | See Table 1 |

Table 1. LC gradient conditions.

| Time (min) | Flow (mL/min) | B (%) |
|---|---|---|
| 0.00 | 0.5 | 0 |
| 15.00 | 0.5 | 50 |
| 15.01 | 0.5 | 100 |
| 20.00 | 0.5 | 100 |
| 20.01 | 0.5 | 0 |
| 25.00 | 0.5 | 0 |

### MS Conditions

| | |
|---|---|
| Instrumentation | Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer |

The MS scan parameters and HESI source parameters are shown in Tables 2 and 3, respectively.

Table 2. MS scan parameters.

| | |
|---|---|
| Scan type | Full MS |
| Scan range | *m/z* 250–2000 |
| In-source CID | 0.0 eV |
| Resolution | 70,000 |
| Polarity | Positive |
| Microscans | 1 |
| Lock masses | - |
| AGC target | 3e6 |
| Max inject time | 200 ms |
| Number of scan ranges | 1 |
| Spectrum data range | Profile |

Table 3. HESI source settings.

| | |
|---|---|
| Sheath gas flow rate | 48 |
| Aux gas flow rate | 20 |
| Sweep gas flow rate | 2 |
| Spray voltage | 3.50 kV |
| Spray current | - |
| Capillary temp. | 400 °C |
| S-lens RF level | 100 |
| Aux gas heater temp | 350 °C |

### Data Processing

Thermo Scientific™ Xcalibur™ Quant software version 3.0.63 was used for data processing. Details are provided in Table 4.

Table 4. Compound transition details used for data processing.

| Sample | Amino Acid Sequence | Precursor *(m/z)* |
|---|---|---|
| Cytochrome C peptide | EDLIAYLK | 483.27301 |
| | GITWGEETLMEYLENPKK | 711.33099 |
| | MIFAGIK | 779.44641 |
| | TGPNLHGLFGR | 390.21155 |
| Leu_Enkephalin | YGGFL | 556.27526 |
| Angiotensin_I | DRVYIHPFHL | 432.8987 |
| Angiotensin_II | DRVYIHPF | 523.77349 |
| Neurotensin | ELYENKPRRPYIL | 558.30907 |

## Results and Discussion

A total of eight peptides were assessed when comparing SOLAµ HRP and filtration as methods of post-digest sample cleanup. Four well-characterized peptides derived from cytochrome C were used for assessment of the SMART Digest kit, selected for their high abundance and stable retention times. Four exogenous peptides were also spiked in post digest. This allowed assessment of the reproducibility of the SMART Digest kit, along with an independent assessment of the cleanup procedures (Figure 1).

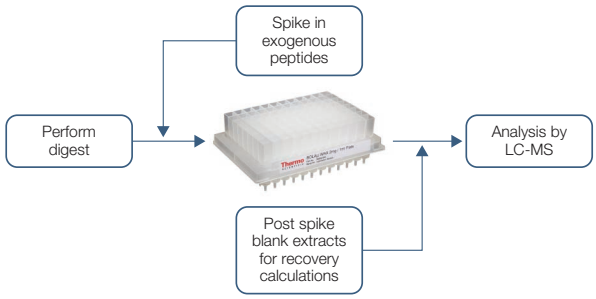



Figure 1. Flow chart of workflow used to calculate peptide recovery from cleanup method.

The separation of all eight peptides is provided (Figure 2). This separation was obtained using an Acclaim C18 RSLC analytical column with a 15 minute LC gradient.

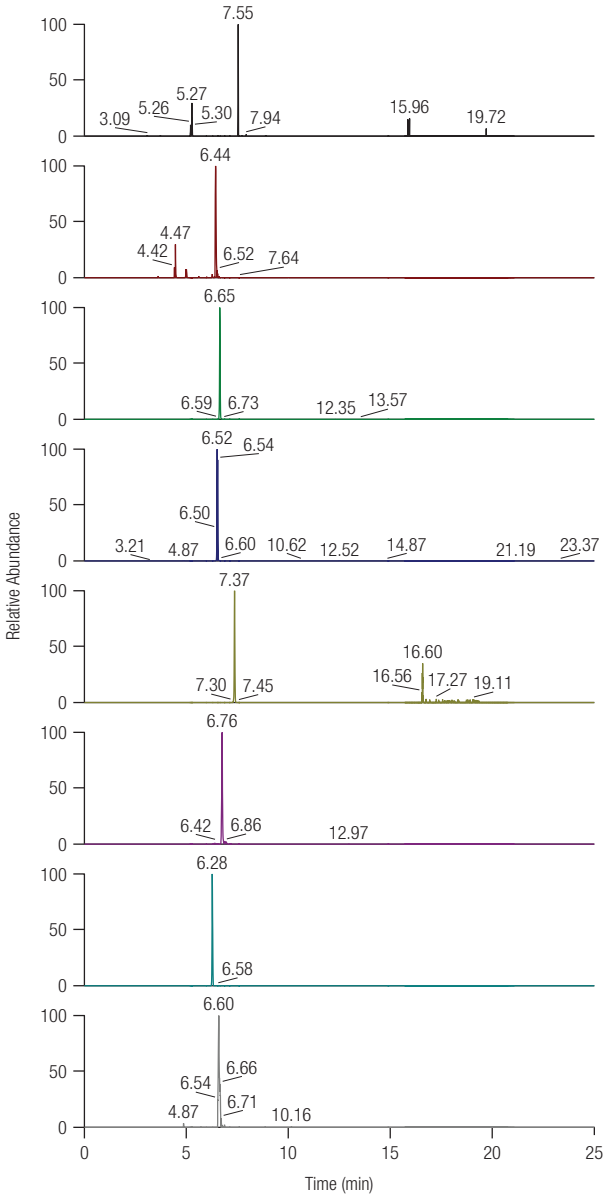



Figure 2. Separation of the eight peptides using an Acclaim C18 RSLC analytical column.

Using the Vanquish UHPLC system, stable retention times were observed for all measured peptides as shown in Table 5.

Table 5. Retention times (RT) of each peptide.

| Sample | Amino Acid Sequence | RT (min) | %RSD of RT (n=6) |
|---|---|---|---|
| Cytochrome C peptide | EDLIAYLK | 7.55 | 0.123% |
| | GITWGEETLMEYLENPKK | 6.44 | 0.186% |
| | MIFAGIK | 6.65 | 0.203% |
| | TGPNLHGLFGR | 6.52 | 0.179% |
| Leu_Enkephalin | YGGFL | 7.37 | 0.127% |
| Angiotensin_I | DRVYIHPFHL | 6.76 | 0.179% |
| Angiotensin_II | DRVYIHPF | 6.28 | 0.156% |
| Neurotensin | ELYENKPRRPYIL | 5.60 | 0.0589% |

Accurate mass data was collected from the Q Exactive Plus mass spectrometer. Mass accuracy was assessed at 500 ppm, 5 ppm, and 2 ppm for assessment. Figure 3 shows the benefits of accurate mass for peptide sequence GITWGEETLMEYLENPKK. Increasing the accuracy of the measurement from 500 to 2 ppm shows clear advantages in reducing background noise levels and ultimately increasing signal-to-noise values of targeted analytes. This also adds further accuracy when measuring unknown samples for identification purposes.

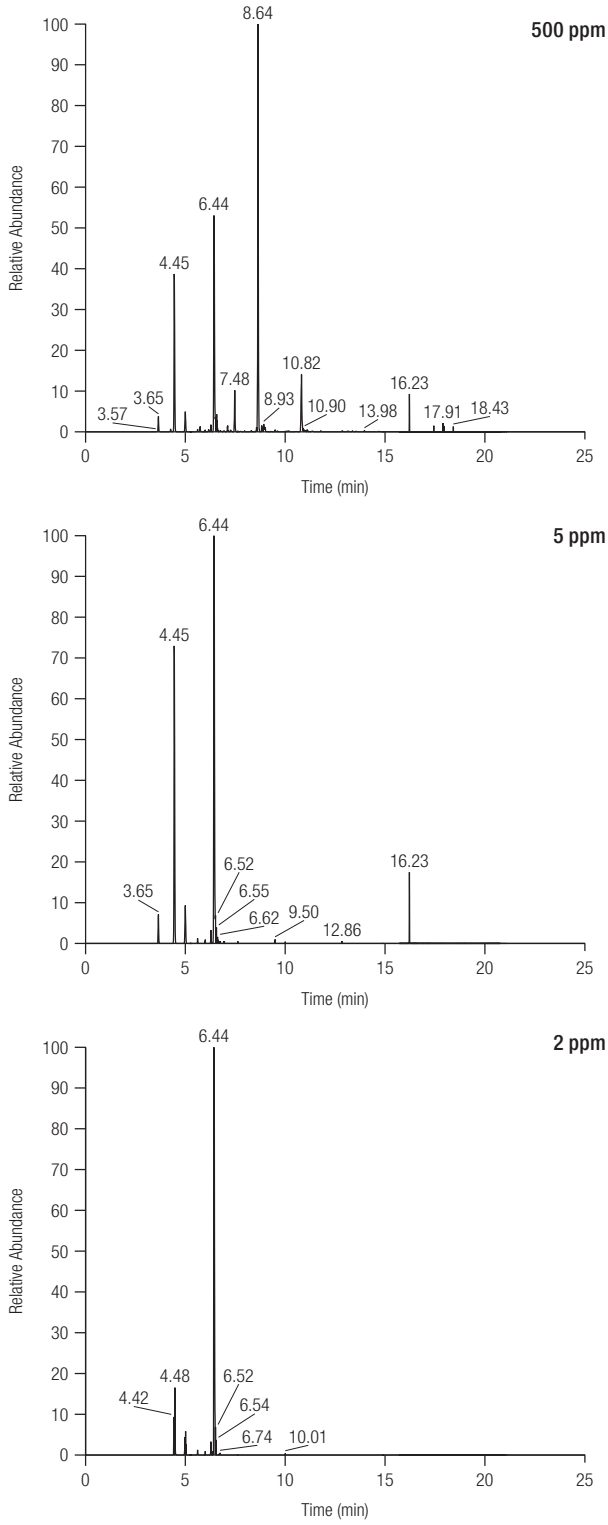



Figure 3. Peptide sequence GITWGEETLMEYLENPKK with accurate mass setting of 500 ppm, 5 ppm, and 2 ppm, RT 6.4 mins.

The recovery and precision of all eight peptides is summarized in Figure 4. This is a direct comparison between SOLAµ HRP and filtration when used as post digestion cleanup methods. The SOLAµ HRP method showed higher levels of recovery on seven out of eight peptides, with higher levels of precision on every peptide.

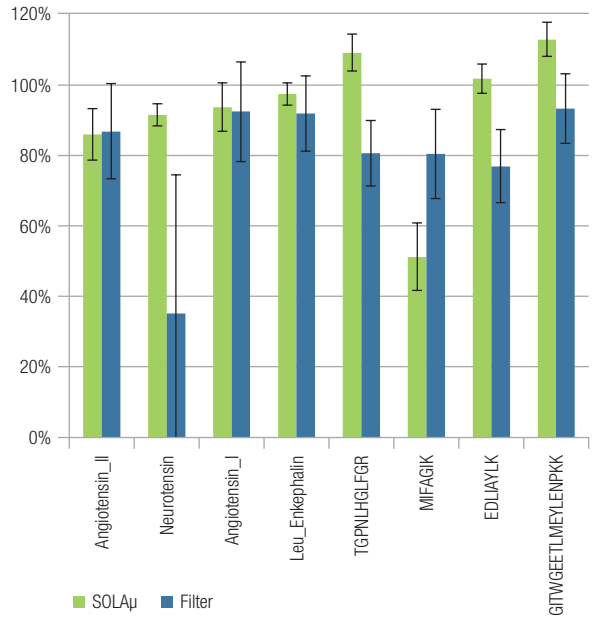



Figure 4. Average recovery values for each peptide with %RSD (n=6).

Some selectivity differences are observed between the two protocols, in particular for neurotensin and MIFAGIK, where recoveries are lower on the SOLAµ HRP for the former and noticeably higher for the latter. Despite the differences in recovery levels, the precision of the data obtained from the SOLAµ HRP is much higher than that obtained with filtration as seen in Figure 5.

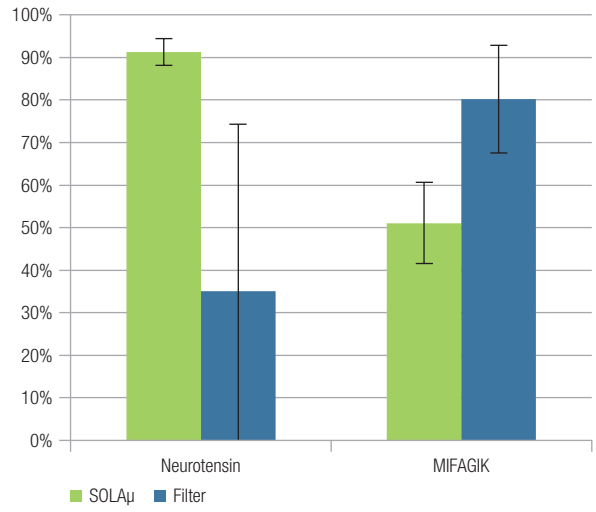



Figure 5. Selectivity differences between SOLAµ HRP and filter.

The percent relative standard deviation (RSD) of the exogenous peptides gives us the level of precision of the cleanup method, along with the subsequent LC and detection. The RSD of the cytochrome C peptides gives us the level of precision of the entire process including the reproducibility of the digest. Comparing precision data between the exogenous and cytochrome C peptides allows assessment of the reproducibility of the digestion (Figure 6).

Comparable levels of precision for both sets of peptides were observed. This indicates the following:

- The entire analytical workflow is precise (RSD of exogenous peptides = 5%)
- The reproducibility using the SMART Digest kit is precise (RSD of cytochrome C peptides = 6%)

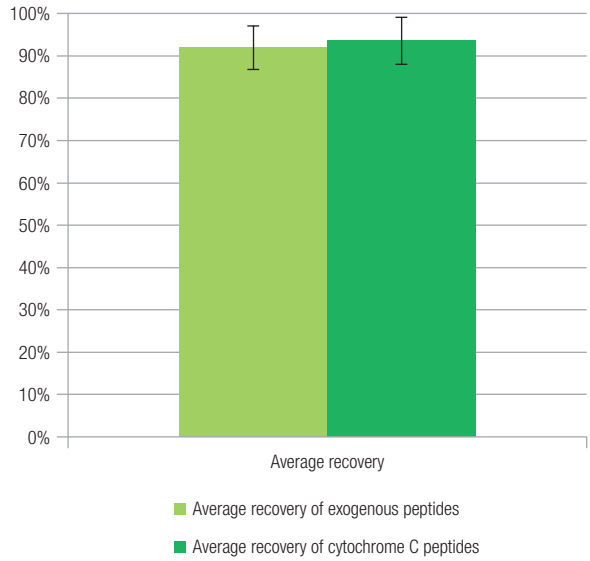



Figure 6. Recovery and %RSD of exogenous and cytochrome C peptides (n=6) both using SOLAµ HRP method.

## Conclusion

This analysis demonstrates that use of the SMART Digest kit offers:

- A highly reproducible digest protocol
- Quick and easy use
- Detergent free digestion

Post digest sample cleanup was achieved using filtration and SOLAμ HRP SPE:

- Filtration provides a simple, fast workflow but offers limited sample cleanup
- SPE provides a high precision workflow with optimized sample cleanup, offering an additional sample concentration factor where required

The benefits outlined above clearly demonstrate the advantages of the SMART Digest kit with regards to workflow efficiency and reproducibility. A choice of sample clean-up is also demonstrated depending on the important factors for the analyst: speed and simplicity, or accuracy and precision of data. The workflow described allows for the introduction of fast, generic, and robust analytical methods within a high-throughput, biopharmaceutical environment.

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