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

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Overview

Here we describe a workflow including novel, rapid and precise digestion of cytochrome C followed by micro-elution solid phase extraction (SPE) clean-up and analysis with next-generation UHPLC and high resolution mass spectrometry detection (UHPLC-HRMS).

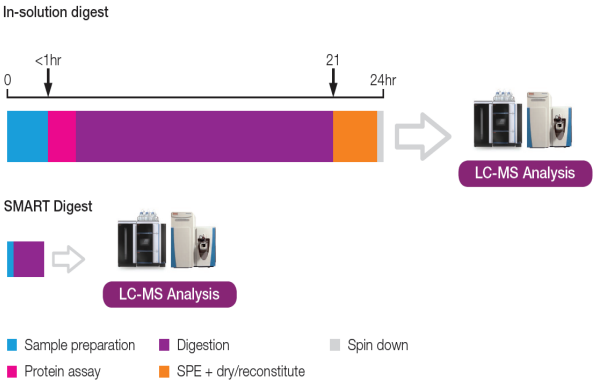
Four well characterized peptides, derived from cytochrome C, were used for assessment of the novel digestion procedure. Four exogenous peptides were also spiked in post-digestion, which allowed assessment of the reproducibility of the digestion along with an independent assessment of the clean-up procedures.

Introduction

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

The Thermo Scientific™ SMART Digest™ kit removes uncertainty associated with conventional solution-based tryptic digestion protocols, resulting in higher reproducibility and higher sample characterization. The immobilized trypsin contained within the kit allows for rapid digest (as quick as 10 minutes for 20 µg of cytochrome C).

FIGURE 1. In-solution digest procedure compared to SMART Digest kit.



The digested sample can either be filtered prior to analysis or cleaned and concentrated with Thermo Scientific™ SOLAµ™ HRP, a highly versatile polymeric micro-scale SPE device offering both polar and non-polar retention of peptides. The ability to elute in low elution volumes reduces the risk of solubility or binding issues as an evaporation step is not required to achieve a concentration factor prior to analysis.

All samples were analyzed using the Thermo Scientific™ Vanquish™ UHPLC system which is optimized to reduce extra column band dispersion. This allows 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 a Thermo Scientific™ Acclaim™ C18 RSLC™ analytical column was achieved within 15 minutes.

Detection was performed on a Thermo Scientific™ Q Exactive Plus™ Orbitrap™ MS, a bench top LC-MS system designed for high-performance, high-throughput screening, compound identification and quantitative analysis. Thanks to its Orbitrap mass analyzer, the Q Exactive Plus system delivers high-resolution, accurate-mass (HR/AM) full-scan MS for fast, precise and reproducible results with analytical confidence.

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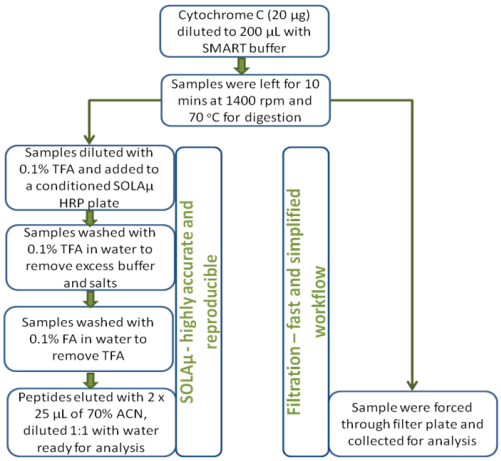
FIGURE 2. Methodologies for the two workflow assessments.



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

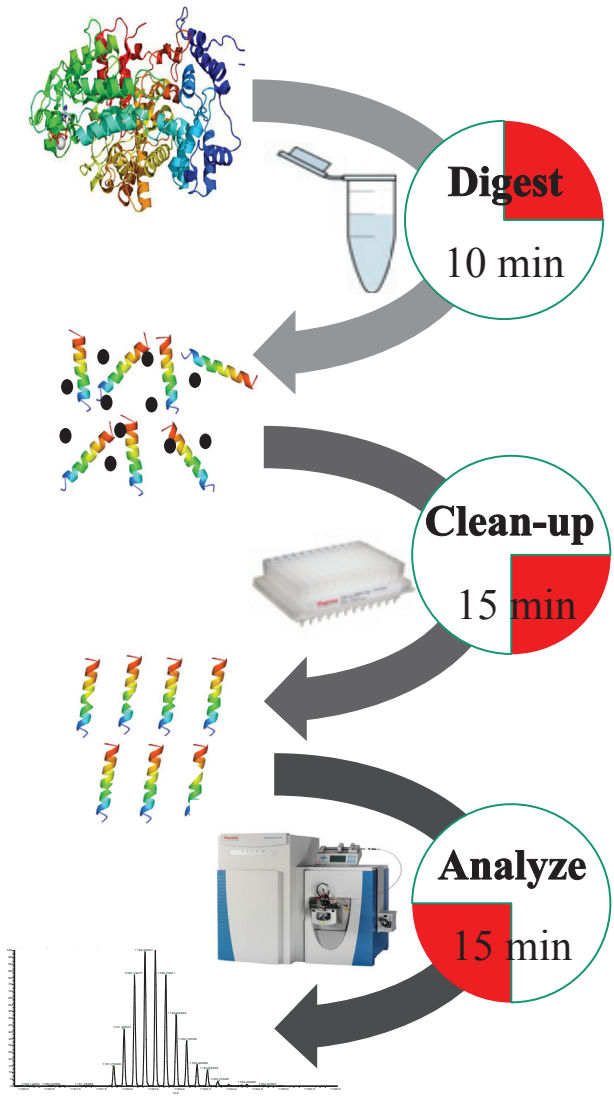
Centrifugation alone cannot always provide the level of clean-up required and exposes the detection system to unwanted levels of contaminants. Consequences can be physical, such as a blocked injection needle, as well as analytical, such as reduced column life or detection variability.

Filtration can be used to prevent the physical issues from occurring but offers little in the removal of excess buffers and reagents. Furthermore, 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 whilst selectively removing the reagents. A generic method can be employed for a non-targeted workflow removing only the unwanted reagents from the digest whilst maintaining high recovery and reproducibility of peptides.

Here we compare two protocols of sample clean-up following protein digestion with the SMART Digest kit; filtration and micro-elution SPE (Figure 2).

FIGURE 3. Workflow using the SMART Digest kit; from protein-to-data within 1 hour.



Methods

A total of eight peptides were assessed (Figure 4), four endogenous cytochrome C peptides and four exogenous peptides spiked in post digestion. Recovery and reproducibility of all eight peptides was measured allowing an assessment of both the SMART Digest kit and the clean-up methods.

FIGURE 4. List of endogenous and exogenous peptides measured as part of the workflow assessment.

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

FIGURE 5. LC-MS conditions used for peptide detection.

Mobile Phase A	0.1% formic acid (water)	AGC Target	3e6
Mobile Phase B	0.1% formic acid (ACN)	Max inject Time	200 ms
Flow rate	0.5 mL/min	Number of scan ranges	1
LC gradient	0 – 50% B over 15 mins	Spectrum data range	Profile
Sheath gas flow rate	48	Mass Accuracy	5 ppm
Aux gas flow rate	20	Scan Type	Full MS
Sweep gas flow rate	2	Scan Range (m/z)	250 - 2000
Spray voltage (kV)	3.50	In source CID	0.0 eV
Capillary temp. (°C)	400	Resolution	70,000
S-lens RF level	100	Polarity	Positive
Aux gas heater temp (°C)	350	Micro-scans	1

Results

The recovery and precision of all eight peptides is summarized in Figure 6. This is a direct comparison between micro-elution SPE and filtration when used as post digestion clean-up methods. The micro-elution SPE method showed higher levels of recovery on seven out of eight peptides, with significantly higher levels of precision for each peptide.

FIGURE 6. Comparison of recoveries and reproducibility of each peptide using the SMART Digest kit with SOLAμ and SMART Digest kit with filtration (n=6).

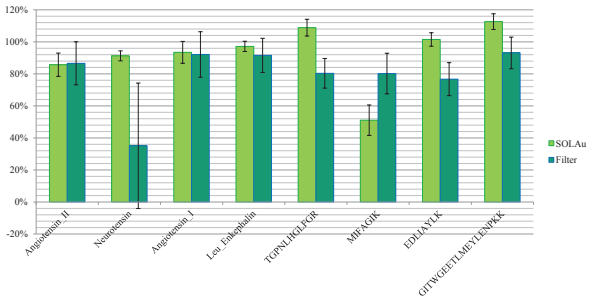
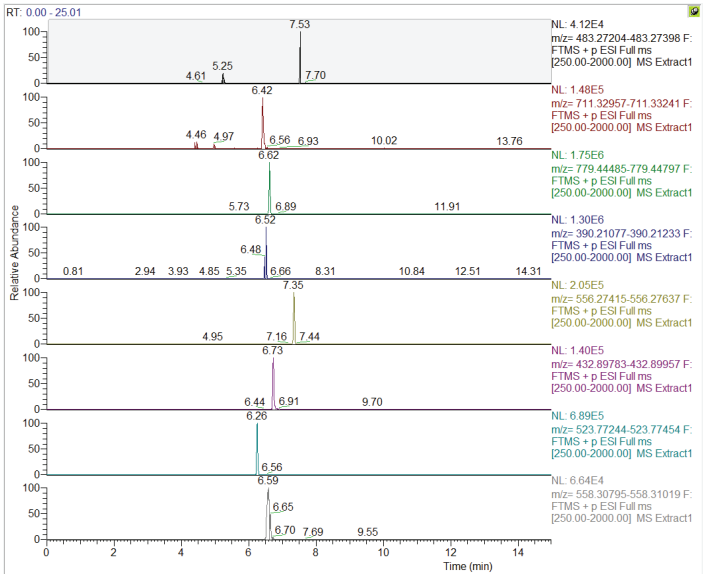


FIGURE 7. Representative ion-extracted chromatogram showing separation of all 8 peptides.



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 clean-up was achieved using filtration and micro-elution SPE;

- Filtration provides a simple, fast workflow but has inherent reproducibility issues.
- SPE provides a high precision workflow with optimized sample clean-up, offering an additional sample concentration factor where required

The benefits outlined above clearly demonstrate the advantages of the SMART Digest kit in conjunction with a choice of sample clean-up depending on the analytical requirements; speed and simplicity, or accuracy and precision of data.

The workflow described allows for the introduction of fast, generic, and robust analytical methods for analysis of peptides within a high throughput, biopharmaceutical environment.

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