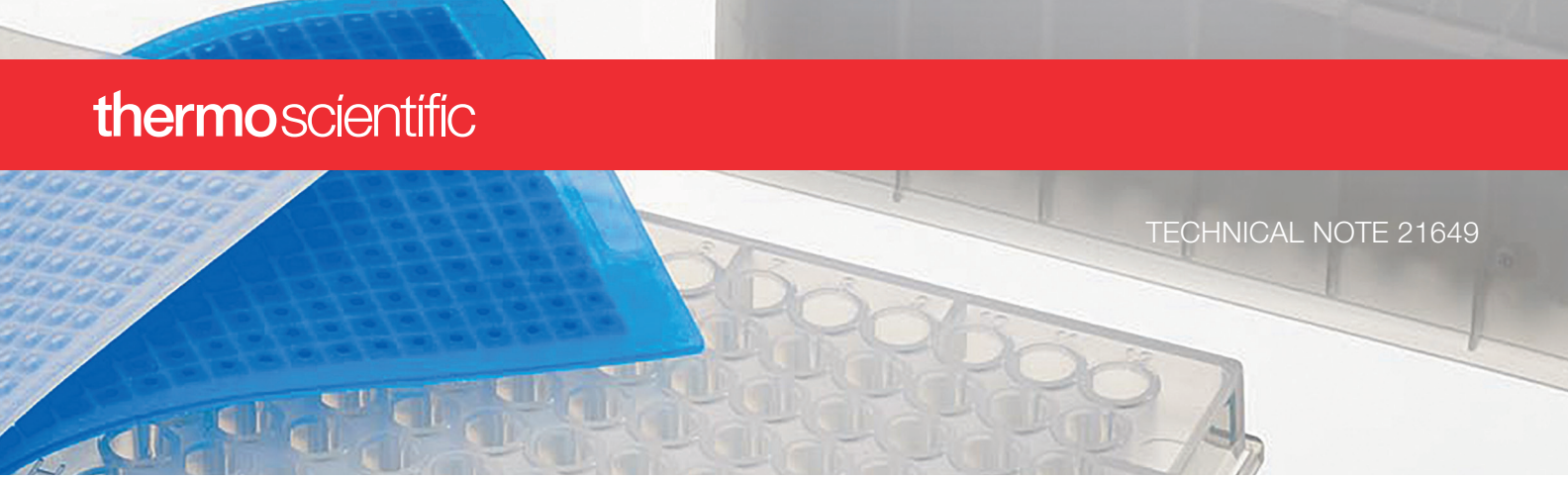


thermo scientific

TECHNICAL NOTE 21649

Recovery study of peptides from different 96-well collection plates

Author

Brian King[1], Detlev Lennartz[2]; [1]Thermo Fisher Scientific, Runcorn, Cheshire, UK; [2]Thermo Fisher Scientific, Langerwehe, Germany

Keywords

Peptides, Digestion, Proteomics

Abstract

The use of 96-well plates for the collection of peptides produced by digestion protocols such as Thermo Scientific™ SMART Digest™ prior to analysis by HPLC depends on the recovery of ever smaller amounts of individual peptides. This study was carried out to determine if there were differences between the recoveries of different peptides caused by the use of plates with different polypropylene grades or glass surfaces/coatings.

Introduction

The use of 96-well plates for collection and handling of protein and peptide solutions in proteomics is becoming more common as many of the processes such a digestion are optimized for smaller amounts of sample as they are frequently used with nano-LC columns and MS detection. Although the miniaturized techniques are capable of working with extremely low concentrations the sample handling within the 96-well plates may be compromised if there is any interaction between the walls of the plates and the sample components. This has been addressed by some manufacturers by the addition of a polar coating onto the surface of the sample plate. This chemical treatment is used by plates where the coating is a polar polymer or by Thermo Scientific™ Plate+™ where the coating is a thin silicaceous layer, analogous to a glass surface. Alternatively, small volume glass tubes have been used for long term peptide storage where a high quality neural borosilicate glass surface gives minimal interaction with hydrophobic peptides.

ThermoFisher SCIENTIFIC

In cases where the sample is a mix of different peptides this approach will ensure better recovery of hydrophobic peptides but may lead to the compromising of more basic polar peptides. It is also possible that intact proteins or larger peptides may show some mix of adsorption.

Conventional techniques make use of uncoated micro plates and tubes for biomolecules without consideration of the surface interaction. The differences in the surface of such plates have been considered with regard to the source of polymer used for the molding of the plates. In many cases the choice of the polymer is not based on purity or surface homogeneity, but the ease of injection molding in terms of speed and temperatures required. Additives are often used to modify the surface properties of the polymer used. The two most common plate materials used are polystyrene and polypropylene. Polystyrene has a high clarity but is harder and more brittle than polypropylene. More importantly in chromatography terms is its limited resistance to long term exposure to organic solvents, especially acetonitrile and THF.

This leaves polypropylene as a solvent stable polymer widely used in the manufacture of a wide range of 96-well plates.

With an untreated polypropylene surface, the chemistry is hydrophobic which may give strong interactions with more hydrophobic peptides and proteins. When used for storage of peptides, untreated polypropylene tubes have been found to give lower recovery of hydrophobic species[1] while glass tubes and treated plastic tubes are more likely to release the peptides when re-solubilized.

Glass vials are often used for biomolecules when carrying out chromatographic analysis. The use of a high quality neutral glass is recommended in order to avoid interaction of basic residues with the acidic silanol groups that are present in greater concentrations on the surface of soda glass commonly used for storage vials and bottles. By selecting vials with a low level of metal oxide components the interactions are largely removed. The 96-well configuration can be used with micro-vials of a diameter of less than 8mm using a 96-position rack or a holding plate. In the case of the Thermo Scientific™ WebSeal™ range of pre-assembled kits the vials are combined with a sealing mat which is effective in sealing the vials, aligning the opening and preventing both contamination and cross-talk between wells. Individual sealed vials may also be used within a block system.

Although compatible with most well plate autosamplers, such plates require modification of autosampler settings in order to withdraw the maximum volume from each well. A solution with a “glassy” surface combined with a standard deepwell plate profile may be preferred for some users.

Options for coating have included the application of organosilicon compounds onto the surface of the collection plates to produce a silicon dioxide layer which is similar to the surface of a silanized glass vial or tube.

The Plate+ Glass Coated Microplate available only from Thermo Fisher Scientific is a high quality polypropylene micro-titer plate treated with an uninterrupted coating of silicone dioxide.

The coating is added to the plate by a proprietary vacuum vapor deposition process that provides complete and uniform coverage of the exposed surfaces.

The Plate+ coating process uses two different organosilicon materials; hexamethyldisiloxane and 1,1,3,3-tetramethyldisiloxane (TMDSO), as the source of silicon. TMDSO and other organosilicon chemicals have been used for many years for the surface deactivation of chromatography columns, vials and other glass surfaces (silanization). A typical silanized glass surface has the organosilicon compound bonding only to exposed surface hydroxyl groups providing a non-continuous coating, one molecule thick. The surface treatment of the Plate+ coating is a continuous layer of deactivated silicone dioxide having a nominal thickness of 200 Å (20 nm).

The surface characteristics of this coating should approximate that of a silanized glass titer plate. The Plate+ surface coating exhibits chemical resistance similar to that of glass.

TMDSO and other organosilicone compounds do not normally adhere to plastic surfaces. The vapor deposition coating process bonds the silicone dioxide to the polypropylene surface of the titer plate. The coating and cleaning process also acts to remove non-bonded traces of the TMDSO. The by-products of the coating process are carbon dioxide and water. The high energy and high vacuum of the vapor deposition process leaves behind a clean, inert, chemical resistant surface.

This provides four possible 96-well plate options.

1. A polypropylene plate of standard manufacture with no treatment and no raw material selection.
2. A polypropylene 96-well plate with no treatment but dependent on the use of a low extraction low-additive raw material using a production molding technique with no chemical mold release agents.
3. A glass vial/tube pre-inserted into a 96-position plate. The glass is a high purity neutral glass with minimal silanol content, but with an amorphous polar surface.
4. A chemical coating onto a polypropylene 96-position plate, which produces a polar surface, and is less retentive of hydrophobic species.

The plates

What is often overlooked is the sealing of the plates once the sample has been introduced. The ability to seal plates used in chromatographic analysis is given by a wide range of injectable sealing mats and covers. The most compatible with chromatographic solvents are those with silicone rubber as their main component. The silicone rubber being an elastomer is able to re-seal after any injection with a narrow gauge needle or probe preventing evaporation of any solvent.

To determine the suitability of different plate types for collection and storage of peptides two comparisons were carried out.

Recovery of an intact peptide: angiotensin

Angiotensin was used as a probe intact peptide and its attachment to different vial and plate surfaces was determined at different temperatures and for extended storage. (See Table 1)

Table 1.

Angiotensin structure						
Sequence (One-Letter Code)	DRVYIHPFHL					
Sequence (Three-Letter Code)	H - Asp - Arg - Val - Tyr - Ile - His - Pro - Phe - His - Leu - OH					
Instrument	Thermo Scientific™ UltiMate™ 3000 RSLC-3 (P/N ULTIM3000RSLCNANO)					
Column	Thermo Scientific™ Accucore™ 2.6 µm C18 50 × 2.1 mm (P/N 16126-052130)					
Method	A: 98:2 Water:ACN + 0.1% TFA					
	B: 10:90 Water:ACN + 0.08% TFA					
Injection Volume	5 µL					
Column Temperature	50 °C					
UV	280 nm					
Autosampler Temperature	Ambient or -4 °C					
Gradient	Time	%B	Flow (mL/min)	Time	%B	Flow (mL/min)
	0	10	0.5	5	95	0.5
	4	40	0.5	5.01	10	0.5
	4.01	95	0.5	6.5	10	0.5

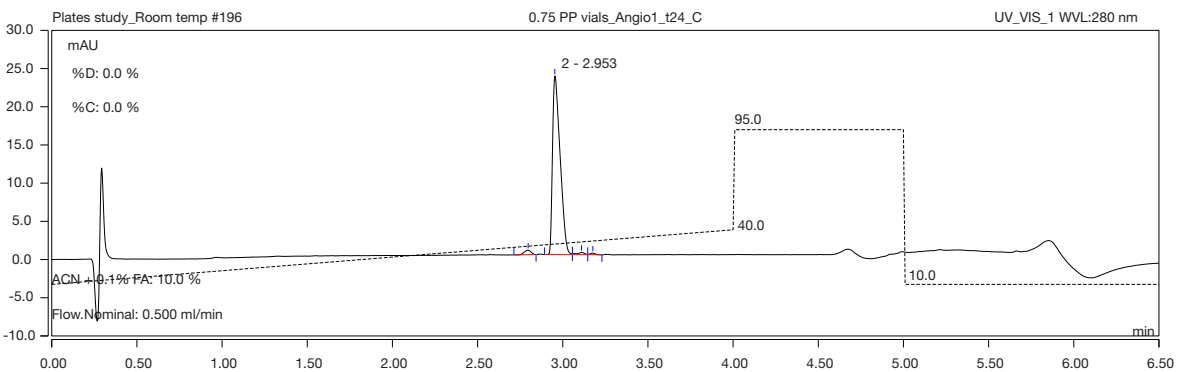


Figure 1. Typical chromatography of angiotensin sample.

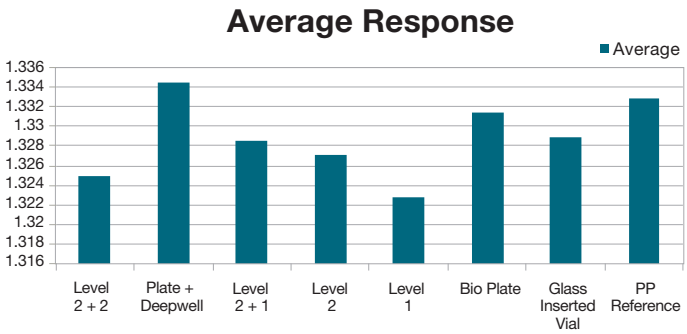


Figure 2. Average amount of angiotensin from the different plates tested.

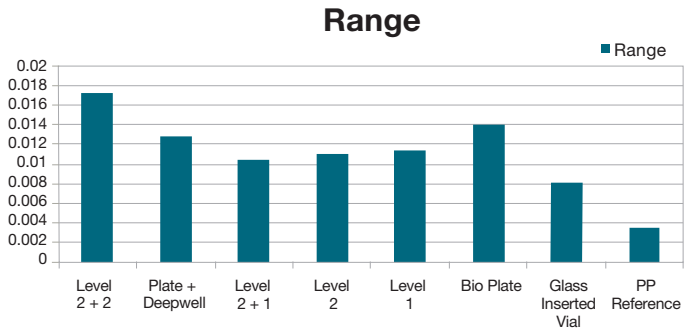


Figure 3. Range of amount measured over 24 hours.

- There were small differences in the reported amount of angiotensin when using the different collection plates
- The standard solution of 0.1 mg/mL was injected from three wells on preparation (t-0) and 2 (t-2), 5 (t-5), 8 (t-8) and 24 (t-24) hours
- Blanks were injected between each plate test

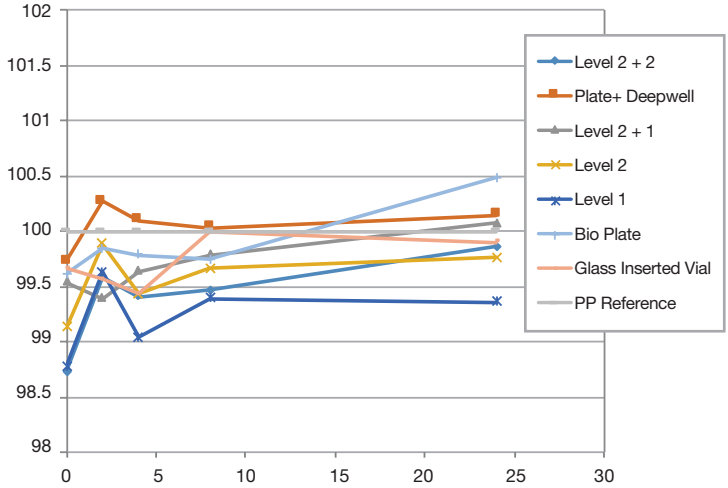


Figure 4. Area measurement for samples stored in the 96-well plates for 24 hours. (Injection through a silicone sealing mat with plug seal.)

- Computed concentration across the plates shows the majority of plates giving very close to 100% for sample concentration
- A number of plates show an effective increase in concentration which may be due to poorer sealing
  - The Plate+ and "Bioplates" overall give the higher signal for the solubilized angiotensin
  - Both of these have a polar coating added to minimize binding of endogenous hydrophobic proteins or peptides
- There were small differences in recovery from the plates evaluated with recovery being stable over 24 hours
- There was no continuing adsorption after 5 hours of storage
- The lowest signal was from an untreated polypropylene Level 1 Plate
- The higher average signal was from a Plate+ glass coated plate
- A selected biocompatible polypropylene plate also gave a high recovery on longer storage

This showed that the angiotensin recovery from the polypropylene plates were largely between 99% and 100.5% for the 100 µ/mL standards. Treatment of the plates can improve recovery, but at these levels the intact peptide levels were similar.

Table 2. Plate identification.

Description	Plate/Vial	Source
Level 2 + 2	1.2 mL Mid Height RB RW	Plate 60180-P133
Plate+ Deepwell	1.2 mL Mid Height RB RW glass coated	Plate+ 60180-P306
Level 2 + 1	1.0 mL Mid Height RW RB certified	CERT Plate 60180-P208
Level 2	2.2 mL Deep Well SQ VB	Competitor 2 plate
Level 1	2.0 mL Deep Well SQ VB	Competitor 1 plate
Bio Plate	1.2 mL Mid Height RW UB	Eppendorf LoBind®
Glass Inserted Vial	0.7 mL Glass Insert, Tapered	Glass Inserted Vial Plate 60180-K101
PP Reference	1.2 mL Mid Height RW UB	CERT Plate 60180-P201

Recovery of peptides produced by tryptic digestion of Cytochrome C

Cytochrome C digestion using SMART Digest

It has been reported that different plates may impact the response from the products of tryptic digestion.[2] To determine if there were differences between an original plate and two certified alternatives a series of digestion using the Thermo Scientific™ SMART Digest™ kits were carried out and initially compared by LC-UV. The use of the SMART digest for the evaluation of endogenous peptides from Cytochrome C digestion had previously shown very good reproducibility and recovery.[3]

Instrumentation

Thermo Scientific™ UltiMate™ 3000 RSLC system equipped with:

- Thermo Scientific™ Dionex™ SRD-3x00 Solvent Racks with Degasser (P/N 5035.9230)
- Thermo Scientific™ Dionex™ UltiMate™ DGP-3600RS Dual-Gradient Rapid Separation Pump (P/N 5040.0066)
- Thermo Scientific™ Dionex™ UltiMate™ WPS-3000TBRS Biocompatible Rapid Separation Well Plate Autosampler (P/N 5841.0020)
- Thermo Scientific™ Dionex™ UltiMate™ TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- Thermo Scientific™ Dionex™ UltiMate™ 3000 Rapid Separation Diode Array Detector (P/N 5082.0020)

Table 3.

Conditions						
Columns	Thermo Scientific Accucore 2.6 µm C18 50 × 2.1 mm (P/N 16126-052130)					
Mobile Phase	A: Water + 0.1 % TFA B: Acetonitrile + 0.08% TFA					
Gradient	Time (min)	A	B	Time (min)	A	B
	0	100	0	15	0	100
	1	100	0	15.01	100	0
	11	50	50	20	100	0
	11.01	0	100			
Flow Rate	0.5 mL/min					
Injection Volume	10 µL					
Column Temperature	80 °C					
Injection Wash	Water + TFA Solvent					
UV Detector	Wavelengths selected were 214 nm and 280 nm					
	Peak width was set to 0.1 min and recommended values were selected for the data collection settings					
Sample Preparation	SMART Digest kit with 96-well filter plate (P/N 60109-102)					

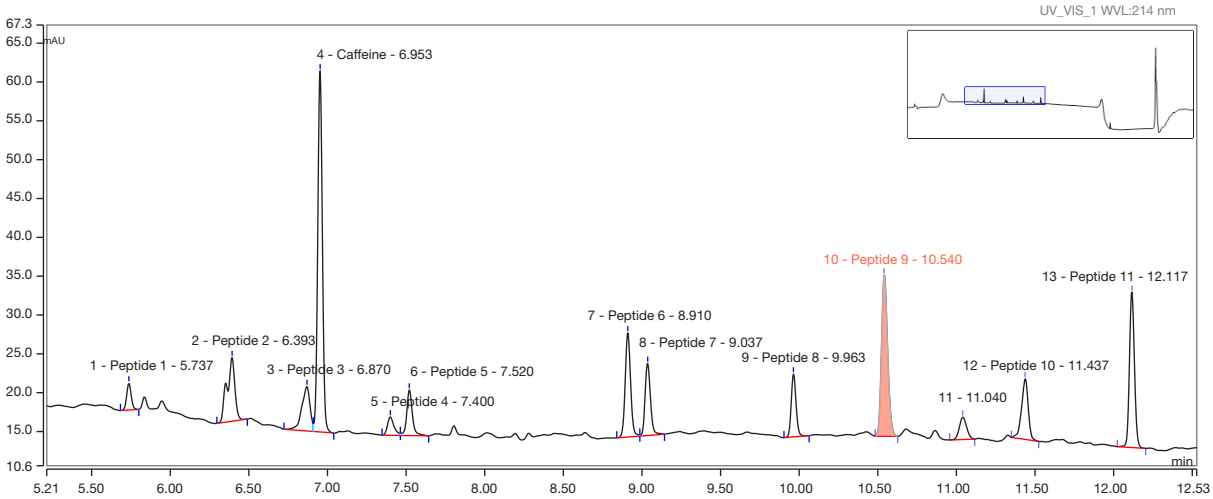


Figure 5. Section of UV chromatogram 214 nm for digest and internal standard solution Plate Level 2 + 1.

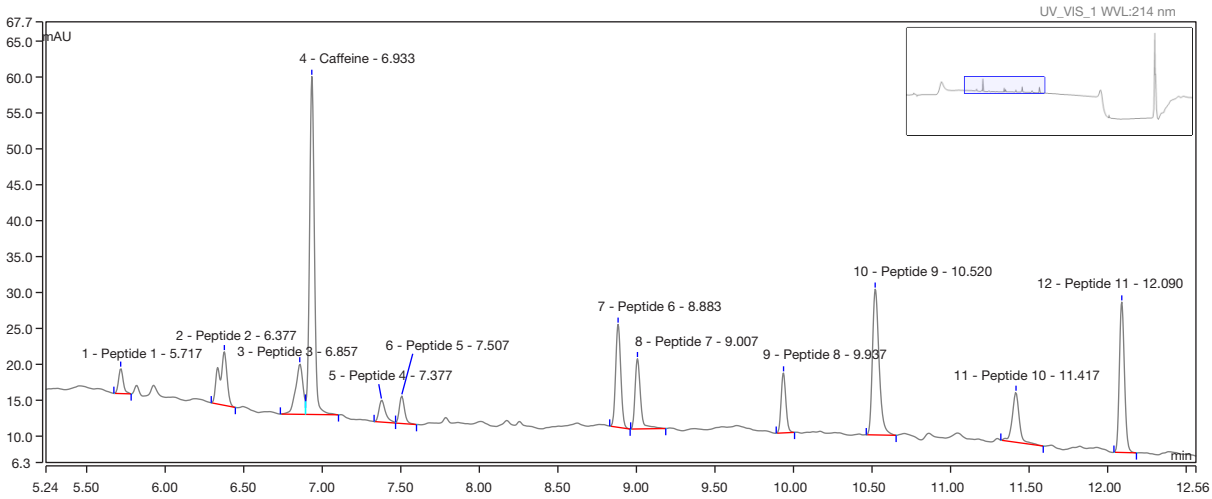
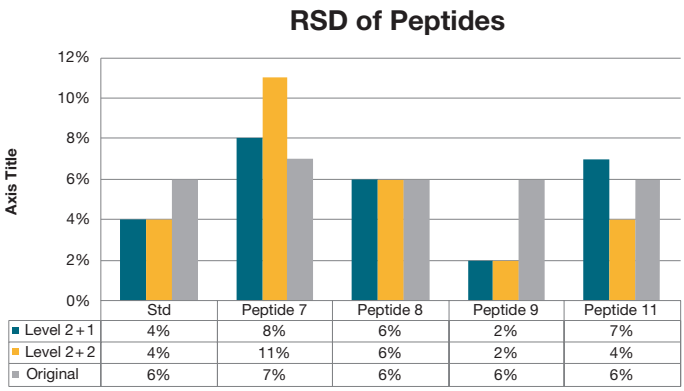


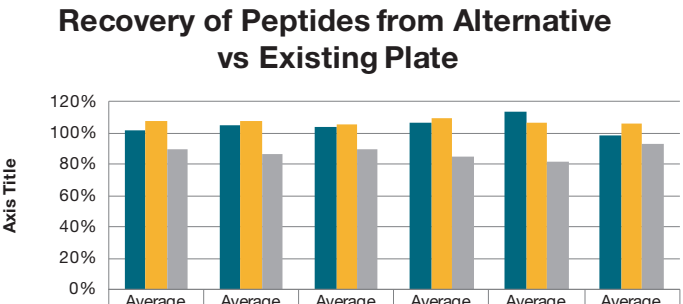
Figure 6. Section of UV chromatogram 214 nm for peptides and internal standard solution Plate Level 2 + 2.



	Std	Peptide 7	Peptide 8	Peptide 9	Peptide 11
Level 2+1	4%	8%	6%	2%	7%
Level 2+2	4%	11%	6%	2%	4%
Original	6%	7%	6%	6%	6%

Figure 7. RSD for internal standard and 4 peptides.

- Peptide 9 showed improved reproducibly over the injection cycles against the existing Thermo Scientific plates while other peptides showed equivalence between the plates
- The Level 2 + 1 Plate showed a more consistent performance against the existing Thermo Scientific plates
- The performance of the more hydrophobic peptides was considered as being most likely to have interactions with the polypropylene surfaces and this was considered likely with later eluting peaks. This was shown particularly with peaks 9–11.



	Average of Area Ratio-6	Average of Area Ratio-7	Average of Area Ratio-8	Average of Area Ratio-9	Average of Area Ratio-10	Average of Area Ratio-11
Level 2+1	102%	105%	104%	107%	114%	99%
Level 2+2	108%	108%	106%	109%	107%	107%
Original	90%	87%	90%	85%	82%	94%

Figure 8. Area Ratio comparison.

Recovery was improved using both plate alternatives. This improvement was not due to any physical or chemical treatment but associated with the material raw material. To determine if this recovery was impacted by the peptide structure a series of digestions were carried out and the peptide solutions stored at fridge temperature 4 °C or ambient temperature. All plates were sealed to reduce possible evaporation impacting signal response.

Recovery of peptides produced by tryptic digestion of Cytochrome C determination by LC-MS/MS

Thermo Scientific™ Vanquish™ Horizon UHPLC binary system consisting of:

- Horizon System Base (P/N VH-S01-A)
- Binary Pump H (P/N VH-P10-A)
- Mixer Kit, 200 µL, VH-P1 (P/N 6268.5120)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active pre-heater (P/N 6732.0110)
- Thermo Scientific™ Viper™ MS Connection Kit for Vanquish System (P/N 6720.0405)

Table 4.

Conditions			
Columns	Thermo Scientific™ Hypersil GOLD™ C18 HPLC (P/N 25003-152130)		
Mobile Phase	A: Water + 0.05% TFA		
	B: Water/Acetonitrile (80:20 v:v) + 0.04% TFA		
Gradient	Time (min)	A	B
	0	95	5
	15.0	45	55
	15.1	0	100
	17.0	0	100
	17.1	95	5
	22	95	5
Flow Rate	0.5 mL/min-1		
Injection Volume	10 µL		
Column Temperature	70 °C, Still air mode		

While the LC-UV data supplied a general measure of individual peptide recovery the use of LC-MS/MS offered the opportunity to identify unambiguously the individual peptides and determine their relative hydrophobicity from the primary sequence. The digestion protocols were repeated with the separation being interfaced to a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer equipped with a HESI-II probe. The instrument was operated in positive ion mode using a spray voltage of 3.4 kV and a capillary temperature of 350 °C (sheath gas pressure 45 psi, S-lens RV level

60 V). Full mass scans were acquired for the *m/z* range of 140-2000 with a resolution of 70000 (FWHM at *m/z* 200; max IT 100 ms, AGC target 3e6).

- The data was acquired with Thermo Scientific™ Xcalibur™ software with instrument control using Thermo Scientific™ Chromeleon™ 7.2.1 SR[4]
- Individual responses for the following peptides were recorded. (See Table 5)

Table 5. Evaluation peptides.

Peptide #	Rt	*m/z*	Charge state	Sequence	Position cleaveage site
6	7.66	1168.62024	1	TGPNLHGLFGR	39
7	7.81	779.44720	1	MIFAGIK	87
8	8.65	964.53484	1	EDLIAYLK	100
9	9.27	545.20921	3	CAQCHTVEK	23
10	10.15	713.68719	3	NA	NA
11	10.84	1005.98053	2	GITWGEETLMEYLENPK	73

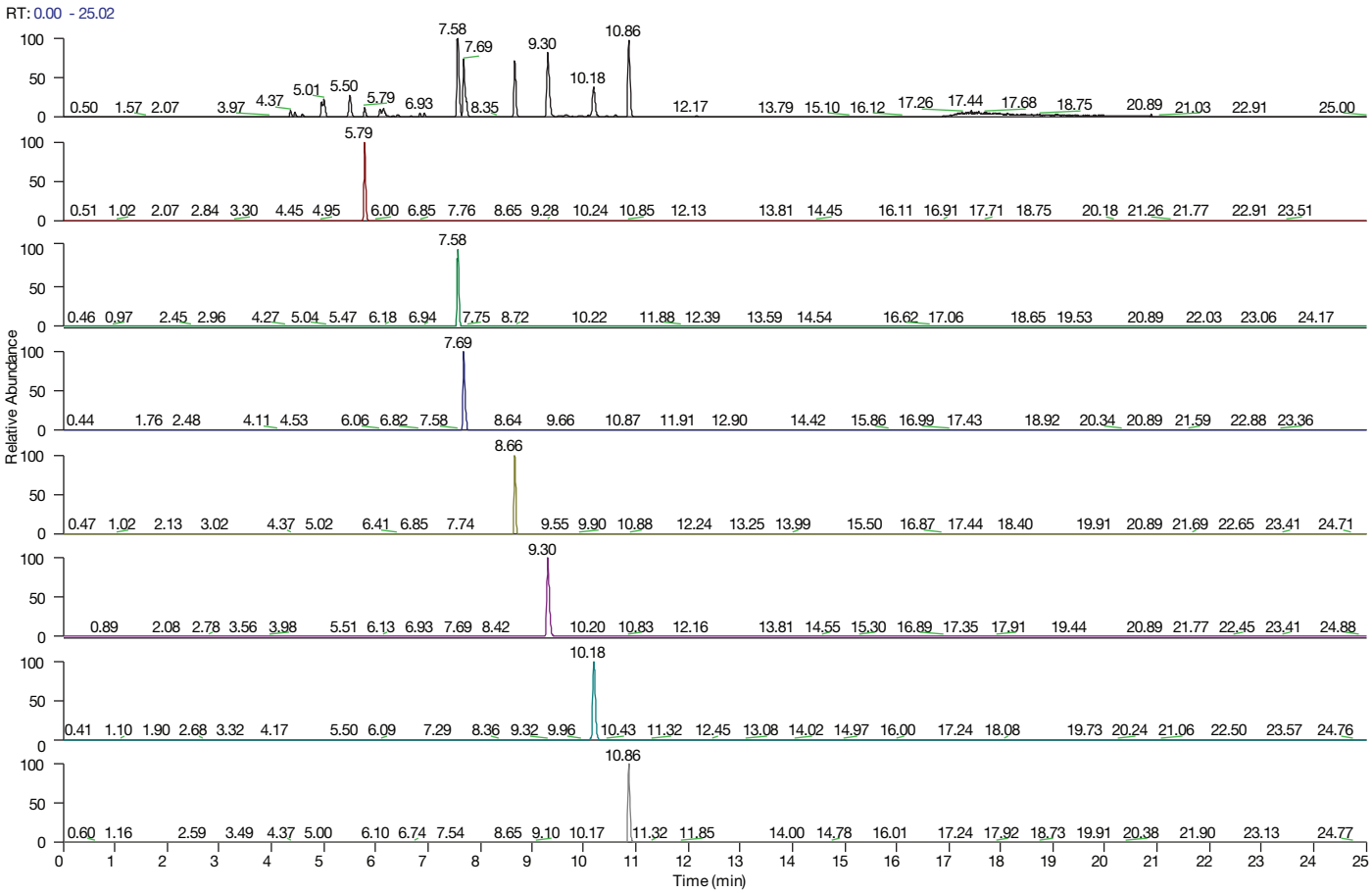


Figure 9. Representative TIC and ion-extracted chromatogram showing separation of all 7 peptides.

Three plates were used as in the previous evaluations.

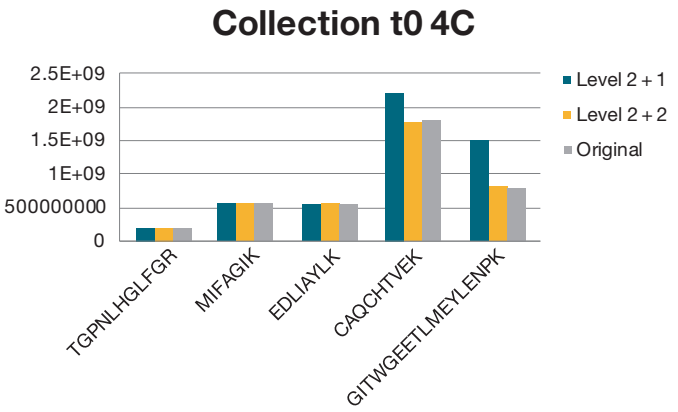


Figure 10. Individual peptide response as prepared.

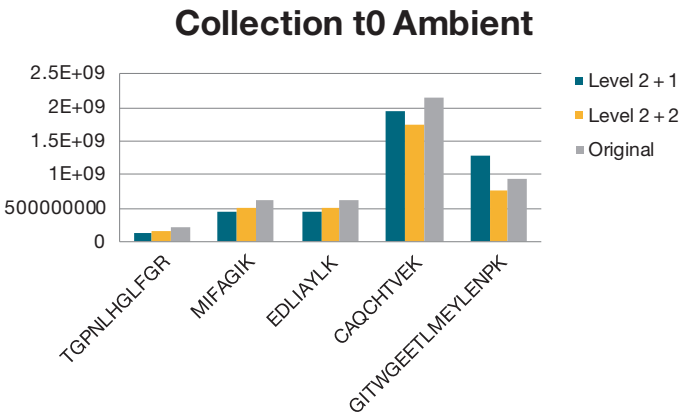
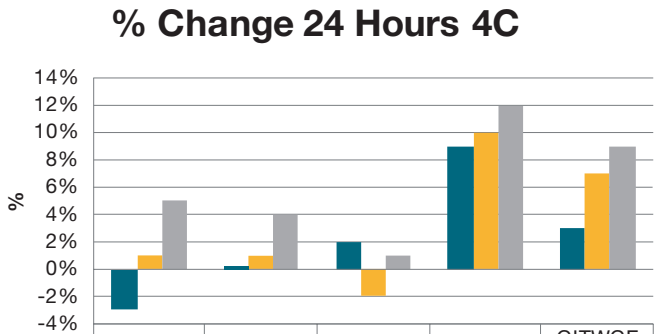


Figure 11. Signal response with collection at ambient temperature into different plates.

- The peptides showed comparable signal for all the measured peptides at 4 °C
- The signal for peptides 9 and 11 were higher on one of the plates. This plate had previously shown higher responses for these peptides.
- At ambient temperature the difference in response was less marked with some additional response seen with peptide 11



	TGPNLHGLFGR	MIFAGIK	EDLIAYLK	CAQCHTVEK	GITWGEETLMEYLENPK
Level 2 + 1	-3%	0%	2%	9%	3%
Level 2 + 2	1%	1%	-2%	10%	7%
Original	5%	4%	1%	12%	9%

Figure 12. Change in response for the identified peptides over 24 hour's storage.

Small changes were seen with the earlier eluting digestion peptides but the more hydrophobic sequence peptide CAQCHTVEK did show some 12% change when stored in the untreated polypropylene plates. This may be reduced with the Level 2 + 1 Plates.

Conclusions

- Collection of products from a 96-well digestion procedure should be securely collected in a complementary collection plate
- The plates showed little evidence of either binding or reduced recovery
- The initial experiment showed that the plates could be selected on the basis of differences in peptide concentration over 24 hours using Angiotensin as a probe
- The selection of two plates with good recovery was then compared to an existing collection plate used for protein digestion using the Cytochrome C digest peptides as models
- The trials showed that improved reproducibility of single peptides was found under these conditions
- The sequence of the peptides were confirmed with LC-MS

thermo scientific

References

1. Alexandra Kraut, Marlène Marcellin, Annie Adrait, Lauriane Kuhn, Mathilde Louwagie, Sylvie Kieffer-Jaquinod, Dorothée Lebert, Christophe D. Masselon, Alain Dupuis, Christophe Bruley, Michel Jaquinod, Jérôme Garinand Maighread Gallagher-Gambarelli *J. Proteome Res.,* **2009**, 8 (7), pp 3778–3785.
2. Bark, S. J.; Hook, V. Differential recovery of peptides from sample tubes and the reproducibility of quantitative proteomic data. *J. Proteome Res.,* **2007**, 6, (11), 4511–6.
3. Jon Bardsley, Joanne Jones, Valeria Barattini, Phillip Humphryes, and Tim Liddicoat Improvement in Speed and Reproducibility of Protein Digestion Utilizing Novel Sample Preparation Technology in a Full Solution Workflow *Thermo Fisher Scientific, Runcorn, UK Poster Note 21209.*
4. van Midwoud P.M., Rieux L, Bischoff R, Verpoorte E, Niederländer H.A. Improvement of recovery and repeatability in liquid chromatography-mass spectrometry analysis of peptides. *J. Proteome Res.,* **2007**, 6, (2), 781–91.

Find out more at **www.thermofisher.com/webseal**

ThermoFisher SCIENTIFIC