[SOLUTION GUIDE]

PEPTIDE BIOANALYSIS

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

“Quickly and effectively develop methods for any peptide... We have built our expertise into the software to provide the tools that will enable you to quickly make the transition from small molecule to peptide bianalysis.”

THE FOCUS ON PEPTIDES IS GROWING

Analyzing peptides may be one of the greatest challenges that the bioanalyst faces at the beginning of the 21st century. Employing LC-MS for the bioanalysis of peptides requires the extraction of the target analyte from a matrix of biochemically similar proteins and peptides. However, we have seen that the most common techniques employed for small molecule extraction, such as protein precipitation (PPT) and liquid-liquid extraction (LLE), do not provide the recovery, sensitivity, specificity, and assay robustness required.

The LC parameters for peptides are very different than those used for small molecules. In a addition, writing a multiple reaction monitoring (MRM) method presents a unique set of considerations when working with peptides, including the presence of multiple precursors and a range of few to many lower abundance fragments.

Here at Waters, we understand the subtleties within each parameter affecting the development of robust, reliable, high sensitivity bioanalytical assays for peptides. We have developed sample preparation protocols and separation conditions that will help you quickly and effectively develop methods for any peptide. Additionally, we have built our expertise into the software to provide you the tools that will enable you to quickly make the transition from small molecule to peptide bioanalysis.

With Waters® solutions, you can have confidence in your results, from tuning your MS all the way through to validating your assays.

Erin Chambers
Principal Scientist
Waters Corporation

BIOANALYSIS OF PEPTIDES

Our aim is to help you master working with large molecules like peptides and understand where methods differ from those for small molecules. Throughout our work with biotherapeutics, we've modified our starting guidelines and identified best practices. This guide shares what we have learned from working with peptides at each point in the workflow.

Peptides vs. Small Molecules

Understand the fundamental differences

MS Tuning and MRM Choice

Learn how to optimize MRMs for peptides and make the best choice for sensitivity and specificity

LC Optimization

Understand the influence of various method development parameters

SPE Method Development

Understand the tools available to obtain the greatest specificity and sensitivity

Peptide Quantification and Data Processing

Tie the whole process together for quantification with easy-to-use processing software

Data Management

Networked – across systems, across laboratories, integrated with your LIMS

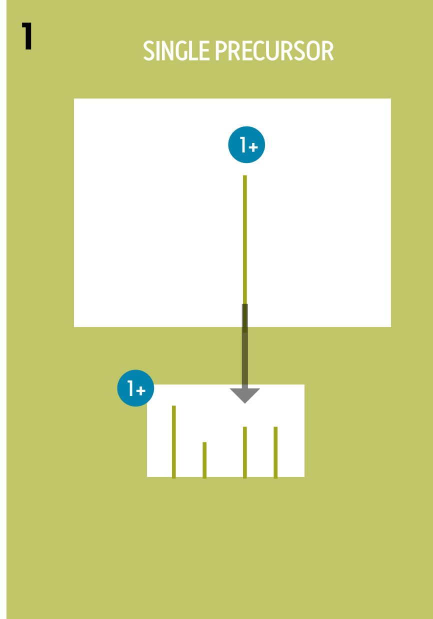
SMALL VS. LARGE MOLECULE–FUNDAMENTAL DIFFERENCES

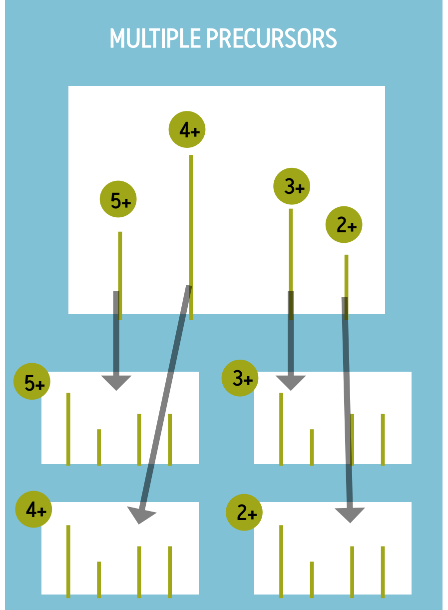
1. **Consider charge state**:
   One peptide might exist in a variety of different charge states. This can dilute the overall ion intensity as the peptide signal is spread across multiple precursor ions.

2. **Pay attention to fragments**:
   The overall MRM signal can be reduced by the sheer number of fragments.

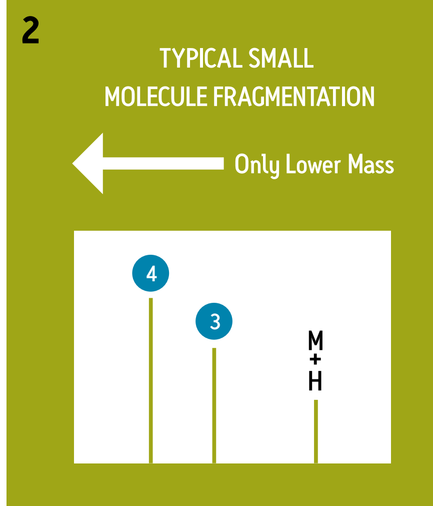
Avoid using water loss or immonium ion fragments, as they are not specific.

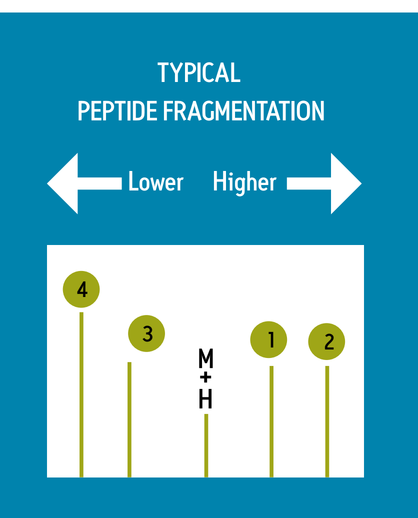
SMALL MOLECULE PRECURSOR | PEPTIDE





Higher *m/z* precursors and fragments typically yield greater MRM specificity.





FOR PEPTIDES, THERE IS MORE TO TRACK

Structures and sequences:

Recording modifications, disulphide bridges, and other sequence information; ensures that any calculations you perform provide the correct information.

Isoelectric point and HPLC index:

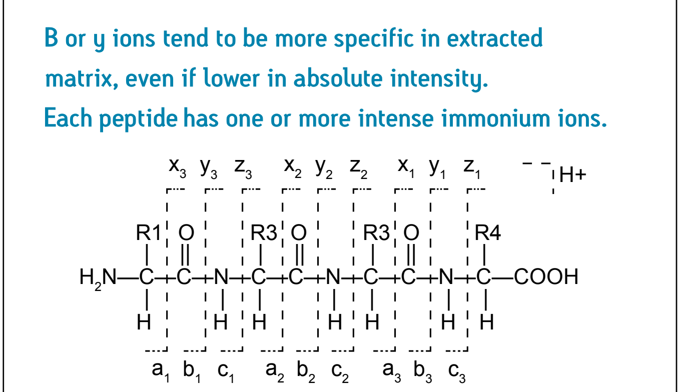
These parameters indicate the pH at which a peptide carries no net electrical charge and a relative sense of hydrophobicity, respectively. Both are useful for developing LC and Solid–Phase Extraction (SPE) methodology.

References, such as papers, methods, COAs, etc.:

To help keep this information handy and tied to your analysis, use a comprehensive Scientific Information System like UNIFI.® UNIFI includes a built–in Scientific Library to store valuable information in one place.

B or y ions tend to be more specific in extracted matrix, even if lower in absolute intensity.

Each peptide has one or more intense immonium ions.

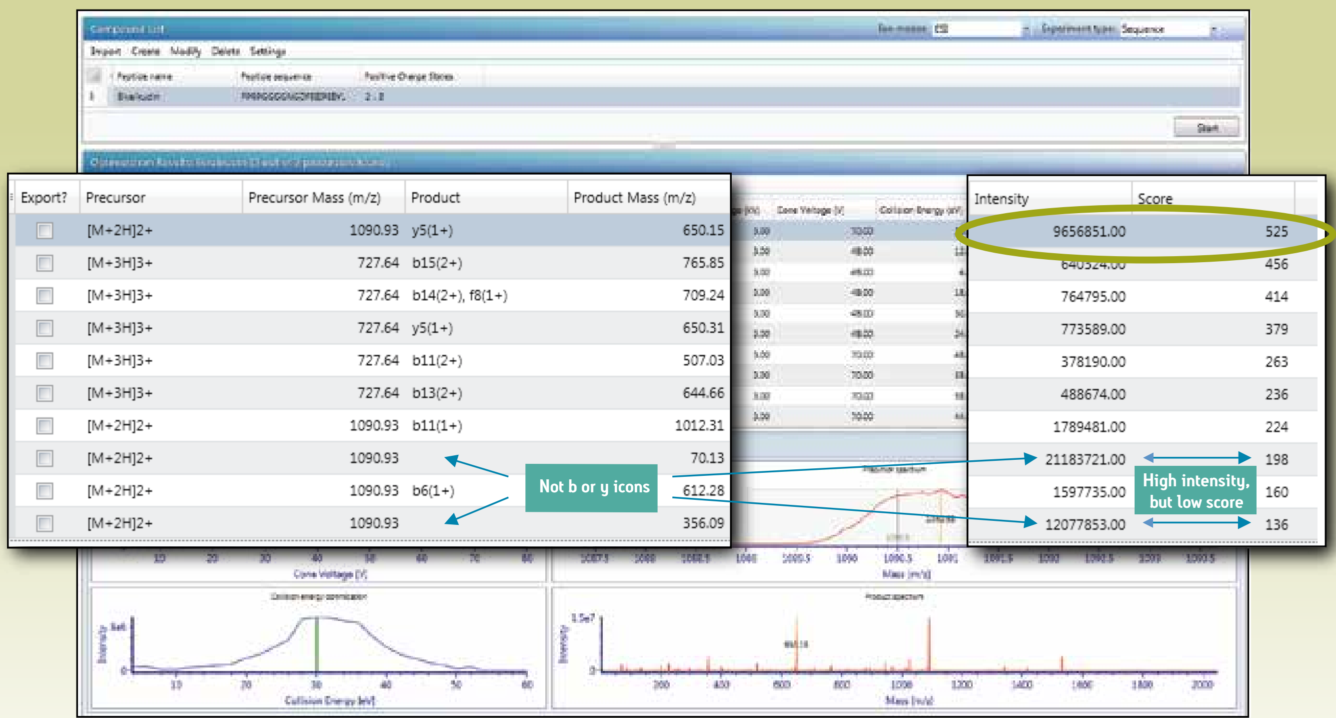


Ensure that you're developing the best MRMs the first time:

When tuning your MS for small molecules, your software probably sorts the MRMs based upon intensity. For peptides, this is not the sensible option; although high intensity, low mass ions are very common in peptides, they typically lack the specificity of higher *m/z* fragments that may correspond to b or y ions.

Compound optimization within UNIFI:

- Calculates the precursors to be optimized
- Performs a thorough MRM optimization.
- Identifies sequence specific ions (b, y, etc.)
- Results are ranked dependent on their mass, intensity, and identification i.e. b/y ion



Export?	Precursor	Precursor Mass (m/z)	Product	Product Mass (m/z)	Intensity	Score
	[M+2H]2+	1090.93	y5(1+)	650.15	9656851.00	525
	[M+3H]3+	727.64	b15(2+)	765.85	640324.00	456
	[M+3H]3+	727.64	b14(2+), f8(1+)	709.24	764795.00	414
	[M+3H]3+	727.64	y5(1+)	650.31	773589.00	379
	[M+3H]3+	727.64	b11(2+)	507.03	378190.00	263
	[M+3H]3+	727.64	b13(2+)	644.66	488674.00	236
	[M+2H]2+	1090.93	b11(1+)	1012.31	1789481.00	224
	[M+2H]2+	1090.93		70.13	21183721.00	198
	[M+2H]2+	1090.93	b6(1+)	612.28	1597735.00	160
	[M+2H]2+	1090.93		356.09	12077853.00	136

Add a carrier protein (0.05% plasma by volume, for example) to solvent and standard diluent to minimize non – specific binding.

LC and column passivation may be necessary when analyzing peptides, as they can retain on untreated surfaces.

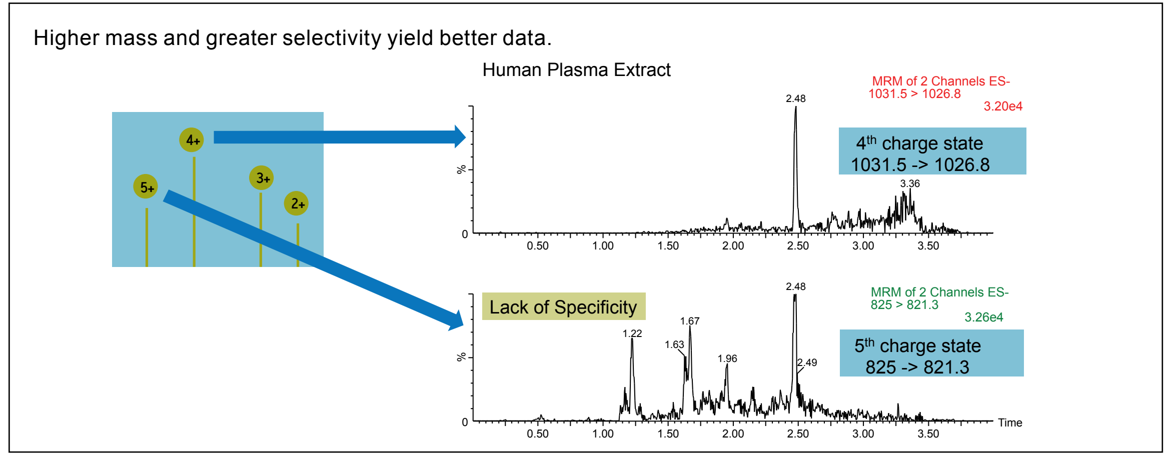
Avoid the use of glass when preparing your samples, as non-specific binding may occur.

A fragment's *m/z* may be higher than it's precursor *m/z*, due to multiple charging.

CHOOSE THE RIGHT MS INSTRUMENT

Why the MS instrument matters

The ultimate sensitivity of your method depends upon an MRM transition that is both sensitive and specific. Generally speaking, higher *m/z* precursor fragments yield better specificity. Therefore, the operating range of the instrument should be able to accommodate large fragment ions and fragment *m/z* values which are higher than the precursor, while providing the highest sensitivity possible within those *m/z* ranges.

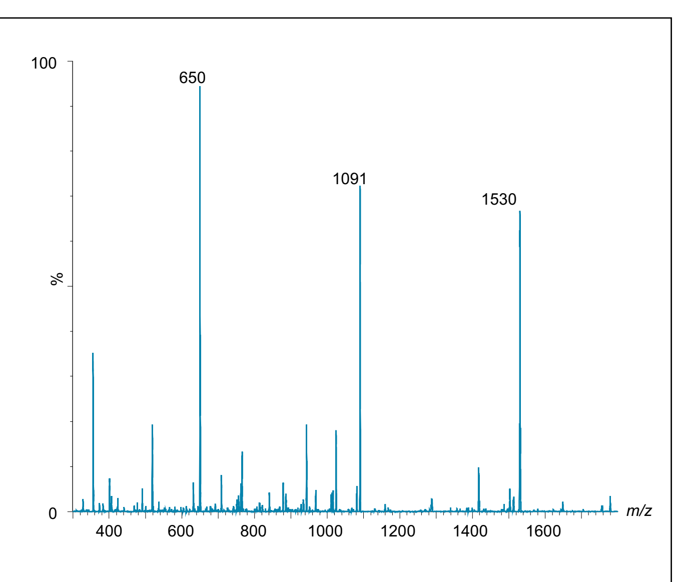


One mass range, one sensitivity

For peptides like bivalirudin, shown below, fragments appear both above the *m/z* of the parent as well as below, thus requiring an MS that can handle a high mass range in both quadrupoles.

If the MS does not have sufficient mass range on both quadrupoles, or an MRM is chosen which lacks specificity, the ultimate sensitivity of the assay will be poor.

The Xevo® TQ-S delivers high sensitivity at high and low mass in the same experiment at the same time, allowing detection of all fragments without compromise.



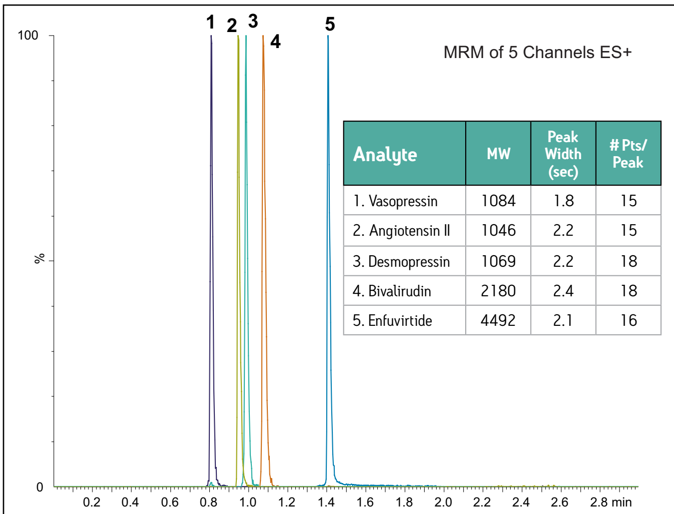
Bivalirudin, MW 2180, *MS scan shows 2+ precursor present at m/z 1091. After performing MS/MS of m/z 1091 from 100 to 1900, major fragments are observed at m/z 650 and m/z 1530.*

KEYS TO SUCCESSFUL METHOD DEVELOPMENT

It is important that a "starting point" method performs equally well for a wide range of peptides including large and small, acidic and basic, hydrophilic and hydrophobic.

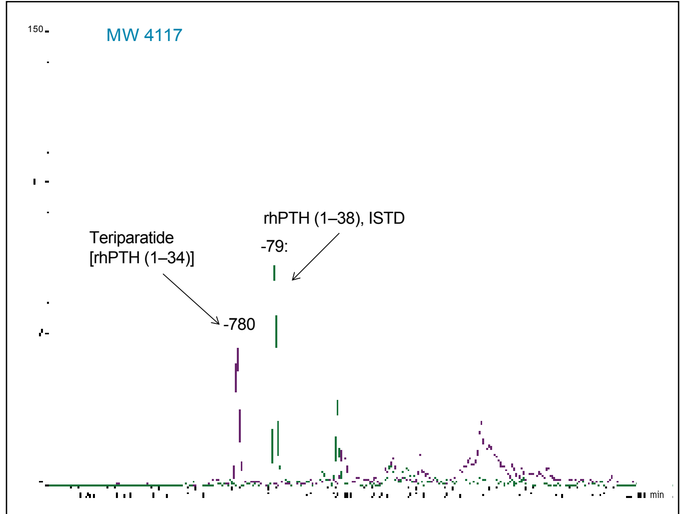
Waters has developed a generic set of chromatographic conditions aimed at providing the best peak shape and separation for a broad range of peptides:
ACQUITY UPLC® BEH C_{18}, 300 Å, 1.7 µm, 2.1 x 50 mm Column, Mobile phase A = 0.1% formic acid;
Mobile phase B = acetonitrile; Flow rate = 0.4 mL/min 5% B to 75% B over 2 minutes; Total run time 3.5 minutes.

Waters recommends screening the ACQUITY UPLC CSH™ and CORTECS® UPLC ® C_{18}+ Columns in addition to the BEH 300 Å using these same gradient conditions.

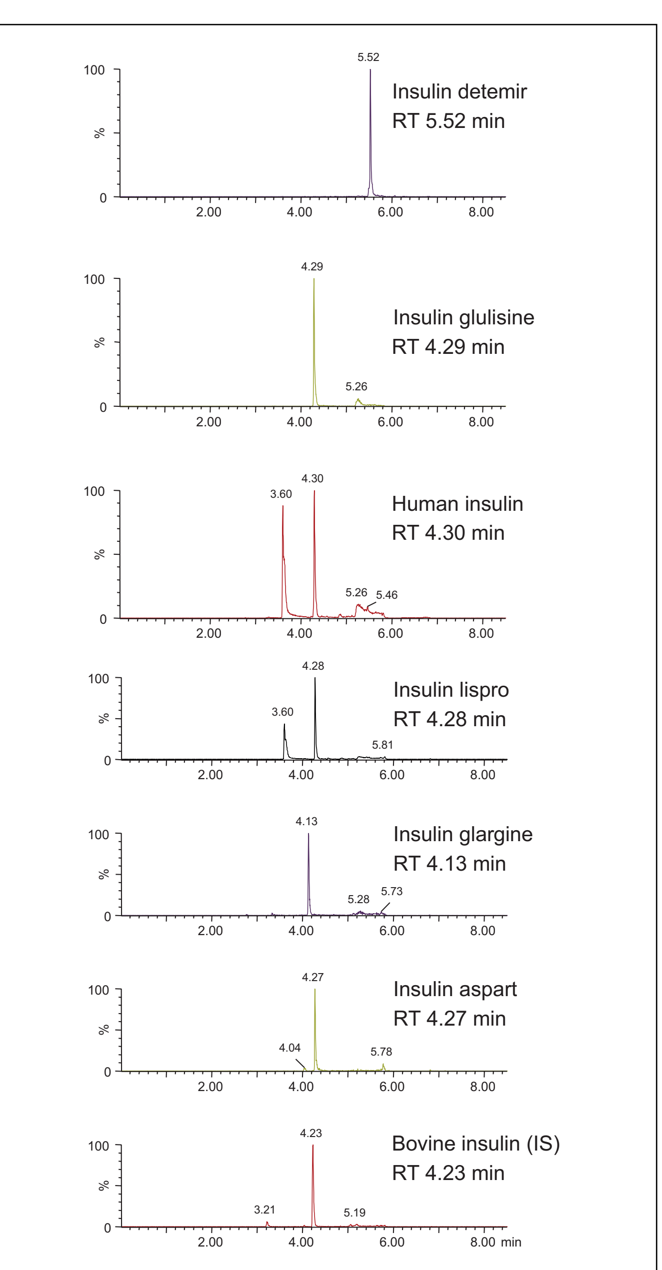


Analyte	MW	Peak Width (sec)	# Pts/ Peak
1. Vasopressin	1084	1.8	15
2. Angiotensin II	1046	2.2	15
3. Desmopressin	1069	2.2	18
4. Bivalirudin	2180	2.4	18
5. Enfuvirtide	4492	2.1	16

Five peptides with diverse properties separated using the generic screening method detailed above.



UPLC-MS/MS separation of teriparatide and internal standard using an ACQUITY UPLC CSH C_{18}, 1.7 µm, 2.1 x 50 mm Column.



UPLC-MS/MS separation of human insulin and 5 analogs using a CORTECS UPLC C_{18}+, 1.6 µm, 2.1 x 50 mm Column.

OPTIMIZING SPECIFICITY OF SAMPLE PREPARATION

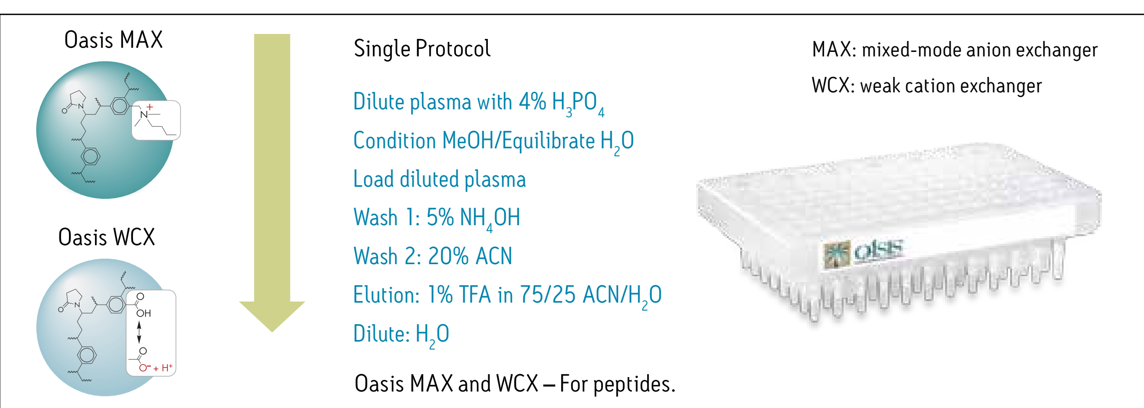
The selective extraction of peptides from other endogenous biological components is probably one of the most challenging sample preparation tasks. The technique of protein precipitation or conventional reversed-phase SPE suffers from significant ion suppression due to matrix effects. The use of highly selective mix mode SPE and a generic screen can help ensure that you extract the peptide from plasma with high recovery and low matrix effects. Mixed mode sorbents have both reversed-phase and ion-exchange retention characteristics, and therefore, may impart orthogonality onto the overall bioanalytical method.

PEPTIDE EXTRACTION WORKFLOW: 2 COMPLEMENTARY MIXED MODE SORBENTS, 1 METHOD

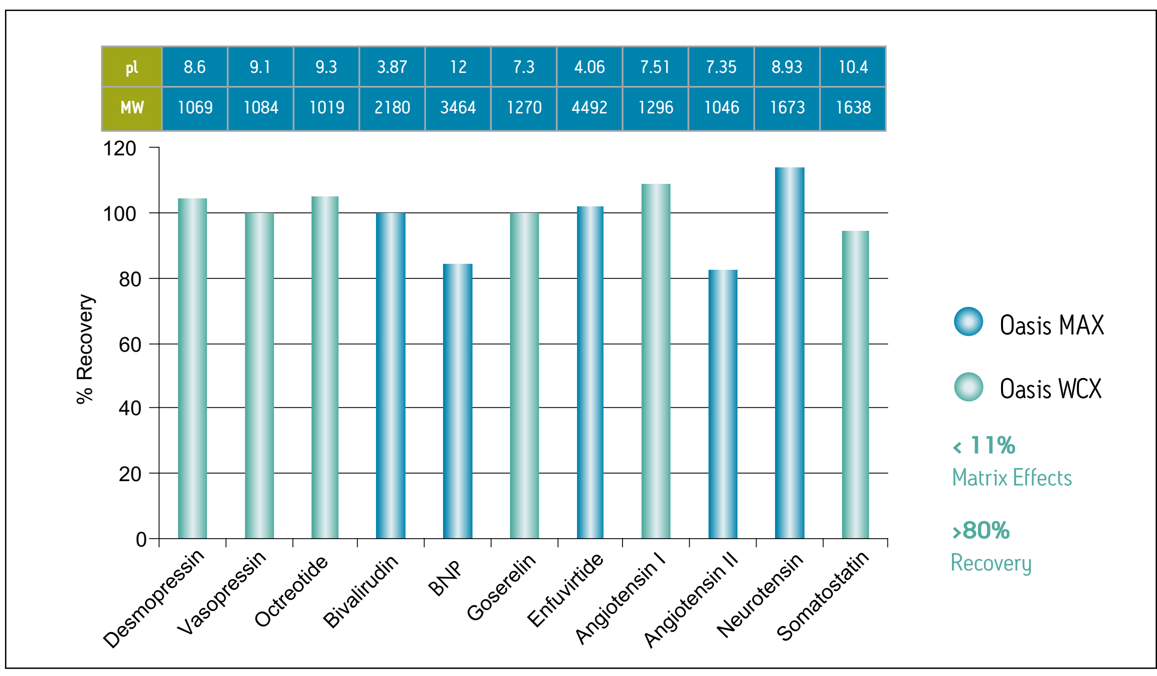
Straight out of the box, using the 2x2 peptide workflow method with Oasis® MAX and WCX SPE μElution Plates, you can achieve high recovery and sensitivity on a wide range of peptides.

Oasis μElution Plates allow for up to a 15-fold concentration of sample.

Aliquot peptide stocks into small volume vials to avoid the freeze thaw cycle which can degrade peptides.



SPE PERFORMANCE FOR PEPTIDES

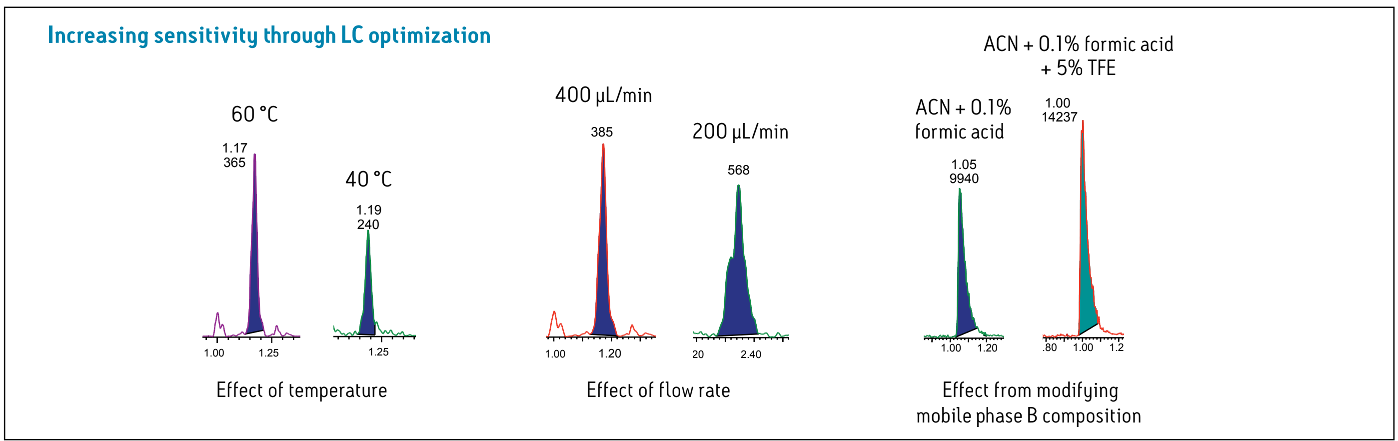


pI	8.6	9.1	9.3	3.87	12	7.3	4.06	7.51	7.35	8.93	10.4
MW	1069	1084	1019	2180	3464	1270	4492	1296	1046	1673	1638

All extracts exhibited >80% recovery and <11% matrix effects using Oasis MAX and WCX μElution Plates. Minor modifications to the generic protocols were made for BNP, Enfuvirtide, and Somatostatin.

METHOD DEVELOPMENT PARAMETERS

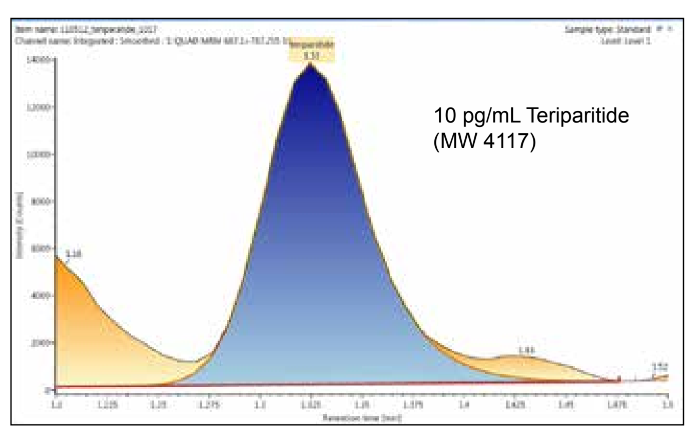
LC and MS source parameters can have a dramatic effect when analyzing peptides. The method development parameters that you should consider testing routinely are mobile phase composition, gradient slope, flow rate, capillary voltage, and column temperature.

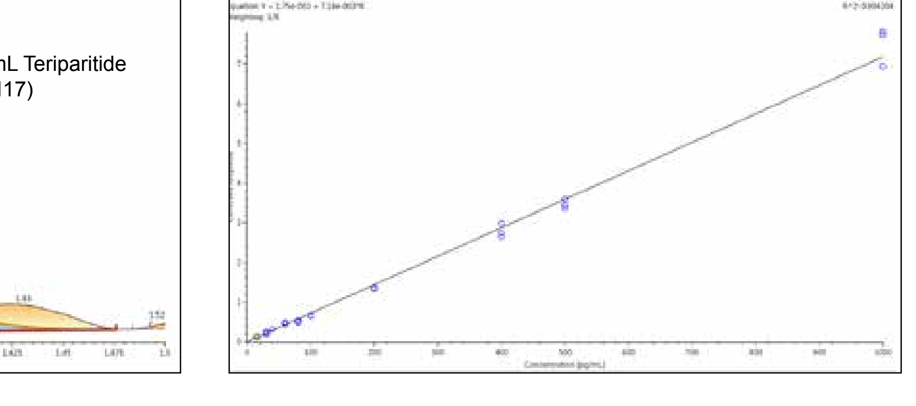


Relative to small molecules, the highest sensitivity for peptides is generally achieved using higher temperatures, lower flow rates, and shallower gradients. In addition, the inclusion of a modifier such as trifluoroethanol (TFE) may help increase sensitivity and/or reduce carryover. To add robustness and reproducibility to the method development process, write a Method Development Template with UNIFI Software. This can then be saved and reused for future biotherapeutic method development.

THE COMBINATION OF SPE, LC, AND MS

When you've put all of these techniques into practice, the results speak for themselves.



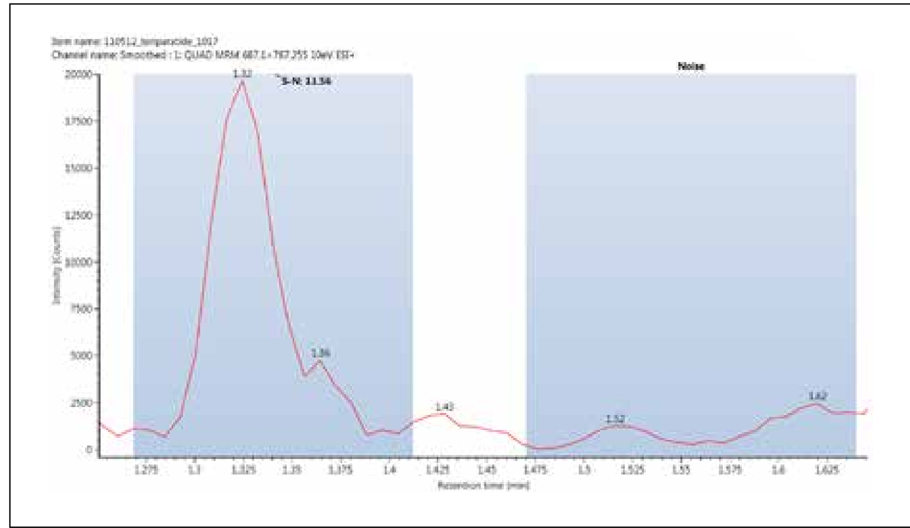


Teriparatide Concentration (pg/mL)	Teriparatide/ IS Ratio	Calculated Teriparatide Concentration (pg/mL)	Mean Accuracy %
10	0.07	10.6	105.6
20	0.14	20.5	102.6
40	0.29	39.0	97.6
60	0.43	57.6	96.0
100	0.73	97.0	97.0
300	2.17	286.4	95.5
600	4.75	626.8	104.4
1000	8.05	1061.5	106.1

Teriparatide QC Concentration (pg/mL)	Mean (N=5) Calculated Concentration (pg/mL)	SD	% CV	Mean Accuracy %
25	25.89	1.32	5.1	103.6
50	51.42	1.91	3.7	102.8
80	83.88	2.15	2.6	104.9
200	202.36	6.49	3.2	101.2
500	511.10	15.23	3.0	102.2

THE SENSITIVITY YOU NEED, WITH SOFTWARE THAT MAKES IT EASY

Software should be designed to make data review and reporting simple. With UNIFI Software, we achieve this by giving you two options for looking at your data, **Investigate** and **Review**:



Investigate can be utilized to easily compare LC-MS methods to assess parameters such as flow rate, gradient profile and mobile phase composition in order to obtain the optimal method for the biotherapeutic of interest.

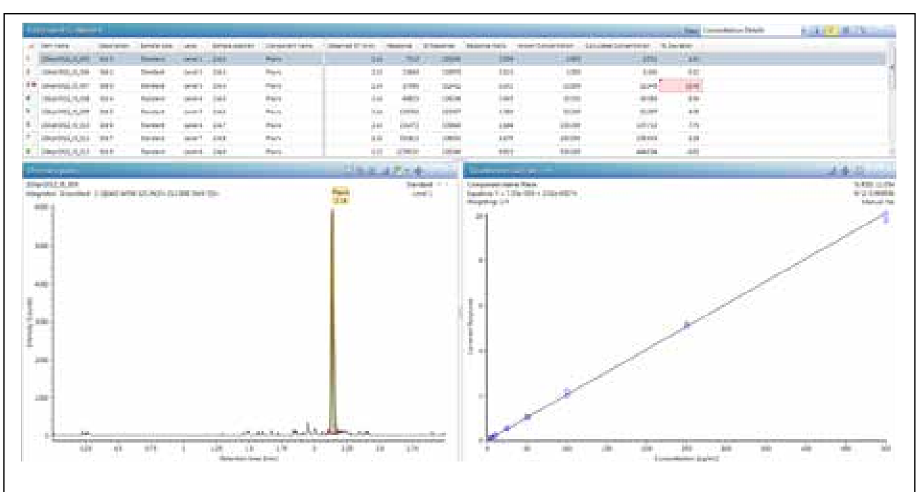
Review displays annotated chromatograms, calibration curves and multiple summary tables, giving the user a way to easily verify the quality of the batch by visualizing critical quality metrics such as:

- signal to noise
- system suitability calculations
- summary statistics such as mean, %RSD

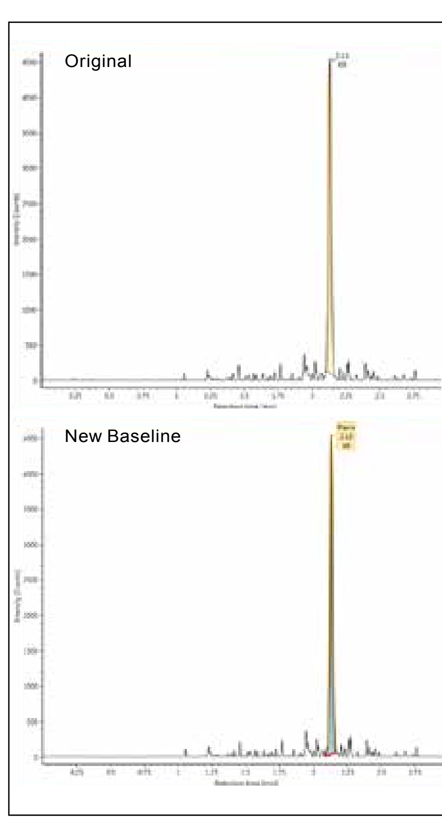
WORKFLOW VIEWS IN UNIFI

The way that you interact with your results is customizable. The workflow steps can be tailored to your individual requirements – building a workflow that works for you.

Quantitative data can be reviewed in a step-by-step manner in order to assess batch performance and to filter and group your results.



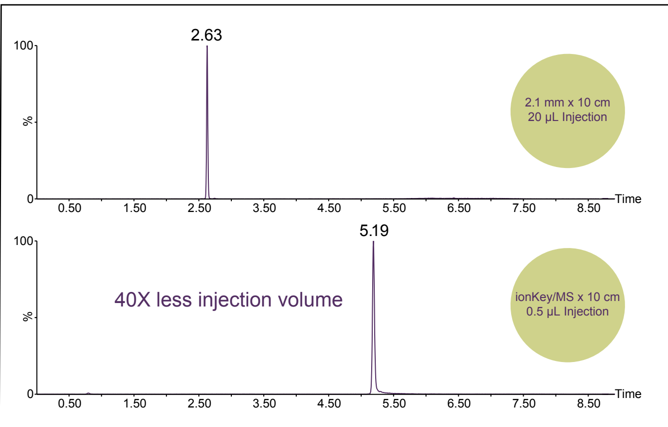
Component Calibration View *quickly shows the user the calibration curve and % deviation to determine curve acceptance.*



Find Manual Integrations View *filters out only samples which have been manually integrated. In addition, the user can view both the modified peak and the original baseline side by side, complete with an Area Manual Change (reported in %).*

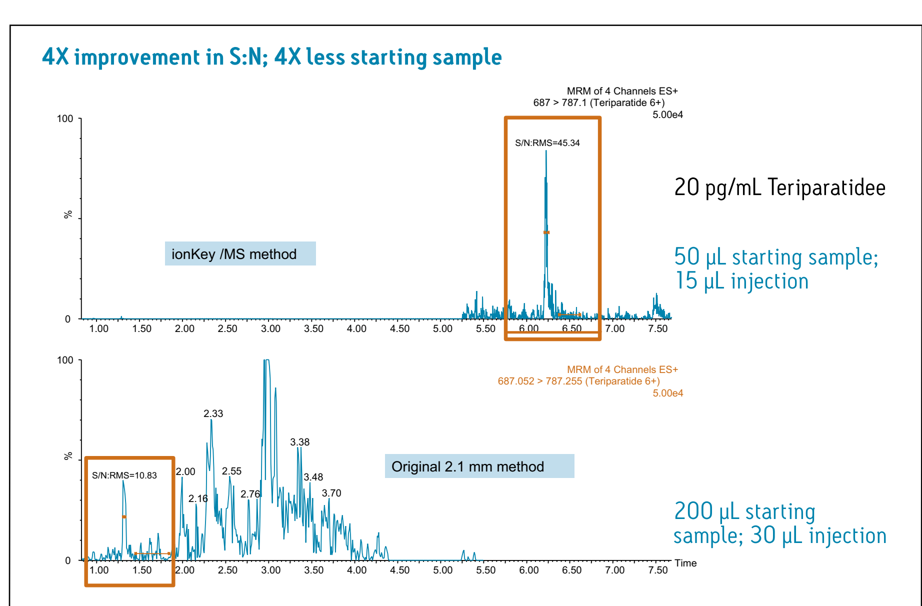
IMPROVED MICROFLUIDICS FOR BETTER SENSITIVITY AND REDUCED SAMPLE LOAD

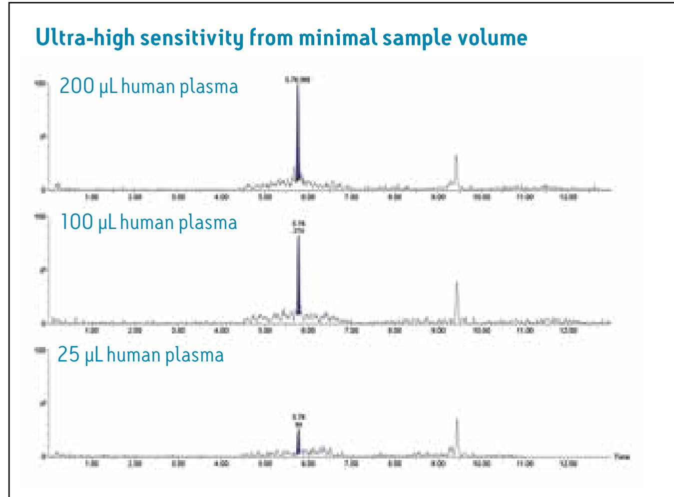
If your method requires additional sensitivity beyond traditional 2.1 mm chromatography, or you need to reduce sample volumes, then you may want to consider the ionKey/MS™ System, an integrated microfluidic system.



Gradient separation of 5 ng/mL of desmopressin standard: ionKey/MS enables scientists to reduce injection volumes and maintain the S:N compared to 2.1 mm I.D. chromatography.

The ionKey/MS System integrates the UPLC analytical separation directly into the source of the mass spectrometer, and the iKey™ Chromatographic Separation Device (150 µm I.D.) contains the fluidic channel, electronics, ESI interface, heater, eCord,™ and the chemistry to perform UPLC Separations. This technology offers significant increases in sensitivity compared to 2.1 mm I.D. chromatography, making it ideal for peptide analyses.





Enhanced sensitivity using ionKey/MS: Extraction Volume Comparison of desmopressin (2.5 pg/mL) from human plasma.

Most bioanalytical LC-MS/MS assays consume high volumes of both solvent and sample, thus increasing the cost /sample and limiting the number of replicates that can be analyzed. In addition to sensitivity improvements, the ionKey/MS technology also affords significant solvent savings and a reduction in sample volume requirements.

Use the Limits functionality in the Analysis Method to easily see failures in the analysis or link to Watson LIMS.

In UNIFI, method development parameters can be automatically adjusted on an injection-to- injection basis, vastly improving the speed of method development.

TOTAL SOLUTIONS FOR PEPTIDE BIOANALYSIS

Waters' solutions for quantitative bioanalysis and bioequivalence are built on logical and optimized workflows that combine robust sample cleanup with fast UPLC Separations; innovative, highly sensitive Xevo tandem quadrupole mass spectrometry; and advanced intuitive software that helps laboratory analysts maintain productivity and compliance.

These user-friendly solutions enable development of highly sensitive and robust bioanalytical assays.

Explore Waters' experience in peptide bioanalysis through the posters, applications notes, and journal citations found on our website: **www.waters.com/peptideBAN**

ACQUITY UPLC

The proven design of the Waters ACQUITY UPLC Systems, used in laboratories for business-critical applications worldwide, ensures higher quality information and dramatically increased productivity.

ACQUITY UPLC H-Class System and UPLC Columns

For routine quantification studies in bioanalysis or bioequivalence

The ACQUITY UPLC H-Class System combines automated quaternary solvent blending with the advanced performance expected of UPLC Separations: high resolution, sensitivity, and improved throughput.

ACQUITY UPLC I-Class System and UPLC Columns

For ultra high sensitivity quantification

The highest performing UPLC ever engineered, ACQUITY UPLC I-Class delivers decreased dispersion for the highest resolution, rapid injection cycles, and sample throughput with reduced carryover, resulting in a clearly superior inlet for any mass spectrometer.

XEVO TANDEM QUADRUPOLE MS/MS

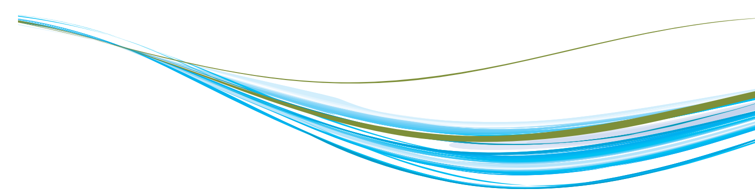
The Xevo TQD, TQ-S micro, and TQ-S are the analytical tools of choice for all of your quantitative UPLC-MS/MS applications, delivering the ultimate in versatility for a wide variety of applications.

ionKey/MS System

ionKey /MS redefines ultimate LC-MS performance by physically integrating the UPLC Separation into the mass spectrometer, delivering a level of sensitivity, chromatographic performance, and ease-of-use that is unachievable by any other LC-MS System.

ionKey/MS integrates with the industry-leading Xevo TQ-S Mass Spectrometer, which enables the lowest detection limits to be achieved for the most challenging analyses, providing users with:

- Increased sensitivity
- Simplified user experience
- Decreased sample volume requirements
- The ability to perform multiple analyses on limited sample volumes
- Reduced solvent consumption



UNIFI

The Regulated Bioanalysis Platform Solution with UNIFI features workflow-driven software that streamlines compliant-ready acquisition, processing, and reporting for UPLC-MS/MS quantification studies for large and small molecules. UNIFI enables the bioanalyst to develop and implement robust methods with high sensitivity requirements. The system and software integrate to transform a laboratory's ability to generate valuable bioanalytical data, and to share that knowledge with collaborators within DMPK organizations, for support of business-critical decisions in drug development.

Scalable configurations include a:

- Workstation supporting a single user at a time
- Workgroup network capable of supporting up to ten users simultaneously.

WATERS ANALYTICAL STANDARDS AND REAGENTS

Waters peptide, phosphopeptide and protein digest standards are high-quality reference standards that help you develop and validate bioanalytical applications performed on UPLC, HPLC, or LC-MS instrumentation. The following standards can be used to develop system suitability.

Description	Part No.	Qty/Box
MassPREP™ Digestion Standards:		
MassPREP Enolase Digestion Standard	186002325	1
MassPREP ADH Digestion Standard	186002328	1
MassPREP E. Coli Digest Standard	186003196	1
Peptide Retention Standard	186006555	1

MASSPREP PEPTIDE MIX

The MassPREP Peptide Standard Mixture contains a void volume (Vo) column marker and nine carefully selected peptides with a broad range of isoelectric points. This standard is useful to test UPLC and HPLC columns and systems dedicated to peptide separations.

Description	Part No.	Qty/Box
MassPREP Peptide Mixture	186002337	1

METHOD DEVELOPMENT KITS

Description	Part No.	Qty/Box
UPLC Therapeutic Peptide Method Development Kit – Includes:	176001835	1
Oasis Peptide µElution Method Development Plate	186004713	1
ACQUITY UPLC BEH 300 C_{18}, 1.7 µm 2.1 x 50 mm Column	186003685	1
96-well 1 mL Collection Plate and Cap Mat	600001043	3
HPLC Therapeutic Peptide Method Development Kit – Includes:	176001836	1
Oasis Peptide µElution Method Development Plate	186004713	1
XBridge® BEH 300 C_{18}, 3.5 µm, 2.1 x 50 mm Column	186003607	1
96-well 1 mL Collection Plate and Cap Mat	600001043	3

CONSUMABLES & COLUMNS

Description	Part No.	Qty/Box
Oasis MAX 96-well µElution Plate	186001829	1
Oasis WCX 96-well µElution Plate	186002499	1
Oasis Peptide µElution Method Development Plate	186004713	1
96-well Sample Collection Plate, 700 µL, Round Well	186005837	25
96-well Sample Collection Plate, 2 mL Square Well	186002482	50
XBridge Peptide BEH C_{18}, 300 Å, 3.5 µm, 2.1 mm x 50 mm Column	186003607	1
ACQUITY UPLC Peptide C_{18}, 130 Å, 1.7 µm, 2.1 mm x 50 mm Column	186003554	1
ACQUITY UPLC CSH C_{18}, 130 Å, 1.7 µm, 2.1 mm x 50 mm Column	186005296	1
CORTECS UPLC C_{18}, 90 Å, 1.6 µm, 2.1 mm x 50 mm Column	186007093	1
CORTECS UPLC C_{18}+, 90 Å, 1.6 µm, 2.1 mm x 50 mm Column	186007114	1

CONSUMABLES & COLUMNS (CONTINUED)

Description	Part No.	Qty/Box
Polypropylene Cap Mat, Round Well for 96–Well Plate	186002484	50
96-well 1 mL Collection Plate and Cap Mat	600001043	1
Extraction Manifold for 96–well Plates	186001831	1
Disposable Reservoir Tray	WAT058942	25
Waters Positive Pressure-96 Processor	186006961	50
Vacuum Box Gasket Kit (includes foam top gaskets and orange O-rings)	186003522	2
SPE Vacuum Pump 115 V, 60 Hz	725000417	1
SPE Vacuum Pump 240 V, 50 Hz	725000418	1

IKEY SEPARATION DEVICES

Description	Part No.	Qty/Box
iKey, BEH Peptide C_{18}, 130 Å, 1.7 µm, 150 µm x 50 mm	186006764	1
iKey, CSH Peptide C_{18}, 130 Å, 1.7 µm, 150 µm x 50 mm	186007257	1
iKey, BEH Peptide C_{18}, 300 Å, 1.7 µm, 150 µm x 50 mm	186006969	1
iKey, BEH Peptide C_{18}, 130 Å, 1.7 µm, 150 µm x 100 mm	186006766	1

SUGGESTED LITERATURE FOR SUPPLEMENTAL INFORMATION

See the following publications for additional information on peptide bioanalysis. In addition, visit **www.waters.com/peptideBAN** for additional references, application notes, posters and presentations.

1. Springer 2013 book: *Characterization of Proteins Using Mass Spectrometry,* editor Guodong Chen. Book chapter "Quantitative Analysis of Therapeutic and Endogenous Peptides Using LC-MS/MS Methods" by Erin Chambers.

2. Chambers EE, Fountain KJ, Smith N, Ashraf L, Karalliedde J, Cowan D, and Legido-Quigley C. Multidimensional LC-MS/MS Enables Simultaneous Quantification of Intact Human Insulin and Five Recombinant Analogs in Human Plasma. *Anal. Chem.*, 2014, 86 (1), 694–702.

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