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Nota applicativa

Development of a SPE LC-MS/MS Method for the Bioanalytical Quantification of Salmon Calcitonin from Human Serum

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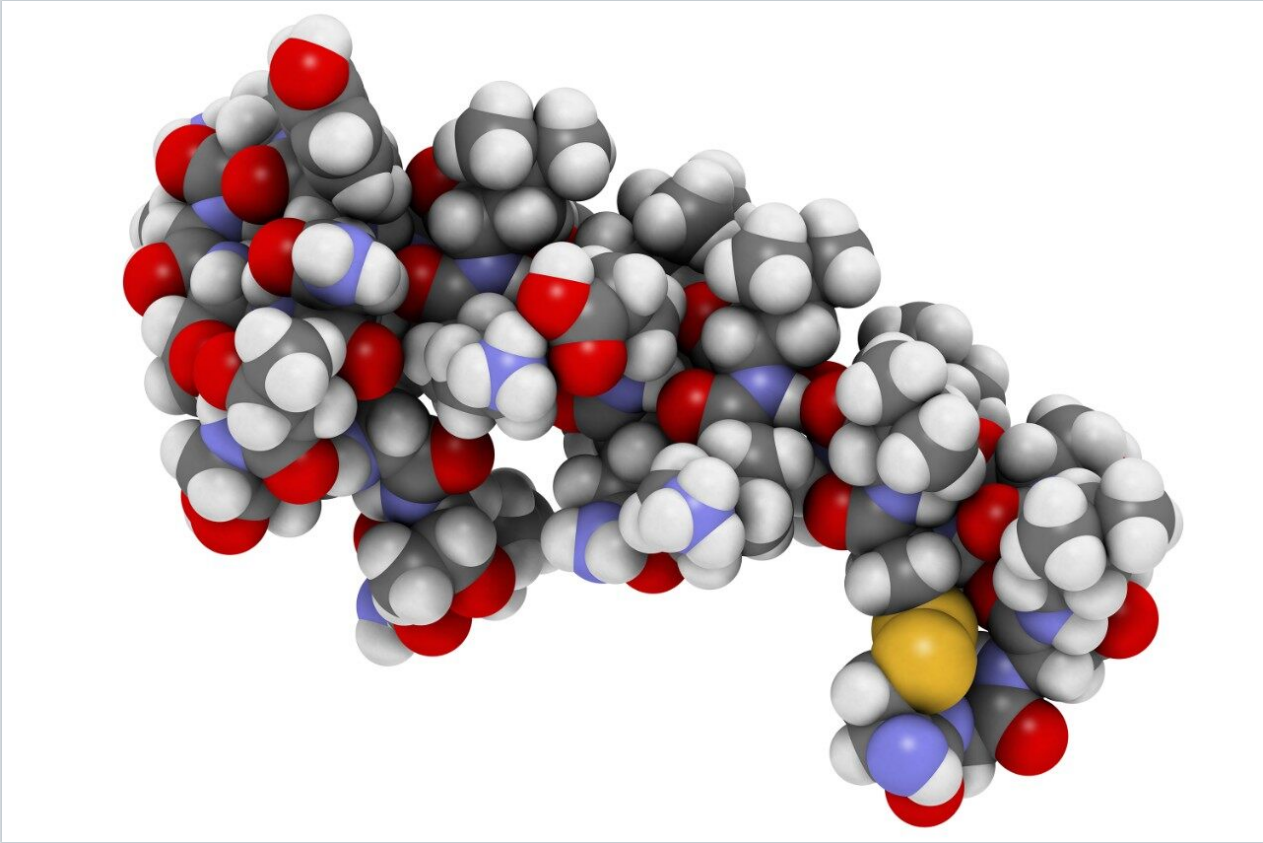

Abstract

This application note describes a simple sample preparation strategy using Oasis WCX µElution SPE combined with analytical flow LC and a tandem (triple) quadrupole MS for the high sensitivity quantification of salmon calcitonin from human serum.

Benefits

- Sensitive and flexible platform for quantification of salmon calcitonin
- Simple, selective, and fast sample preparation using Oasis mixed-mode SPE in µElution format
- Intact quantification without sample digestion, reducing time and variability
- CORTECS UPLC Columns for high sensitivity and narrow peak widths
- High sensitivity and accuracy using UPLC separation and Xevo TQ-XS Mass Spectrometer

Introduction

Salmon calcitonin (sCT) is a 32 amino acid (MW 3439.1 Da) synthetic polypeptide and is used in the treatment of Paget's disease, osteoporosis, and hypercalcemia.[1] Its amino acid sequence and structure are highlighted in Figure 1.[2] Accurate quantification of sCT can be challenging, as the pharmacokinetics of sCT is characterized by rapid absorption within 30 minutes and rapid elimination with a half-life of ~1 hour, resulting in very low circulating plasma levels (pg/mL).[2,3] Although peptide biologics like sCT have historically been quantified using ligand binding assays (LBAs), over the past few years, there has been a growing trend towards the bioanalysis of large molecules by LC-MS. This is, in part, driven by the fact that LBAs can suffer from cross-reactivity, lack of specificity, limited dynamic range, and long method development times. In contrast, LC-MS has the advantage of shorter development times, greater accuracy and precision, the ability to multiplex, and can readily distinguish between closely related analogues, metabolites, or endogenous interferences. Previously described LC-MS methods for salmon calcitonin have achieved a lower limit of quantification (LLOQ) of 50 pg/mL by tuning the tandem (triple) quadrupole to achieve a resolution of 0.2 Da as opposed to the unit resolution at which these instruments usually operate,[3,4] while other methods have used larger sample volumes (500 µL) to achieve similar LLOQ's. The work described here uses UPLC separation, tandem (triple) quadrupole MS and simple, fast, and selective sample

preparation in a 96-well format to achieve an LLOQ of 25 pg/mL, extracted from 100 µL of serum.

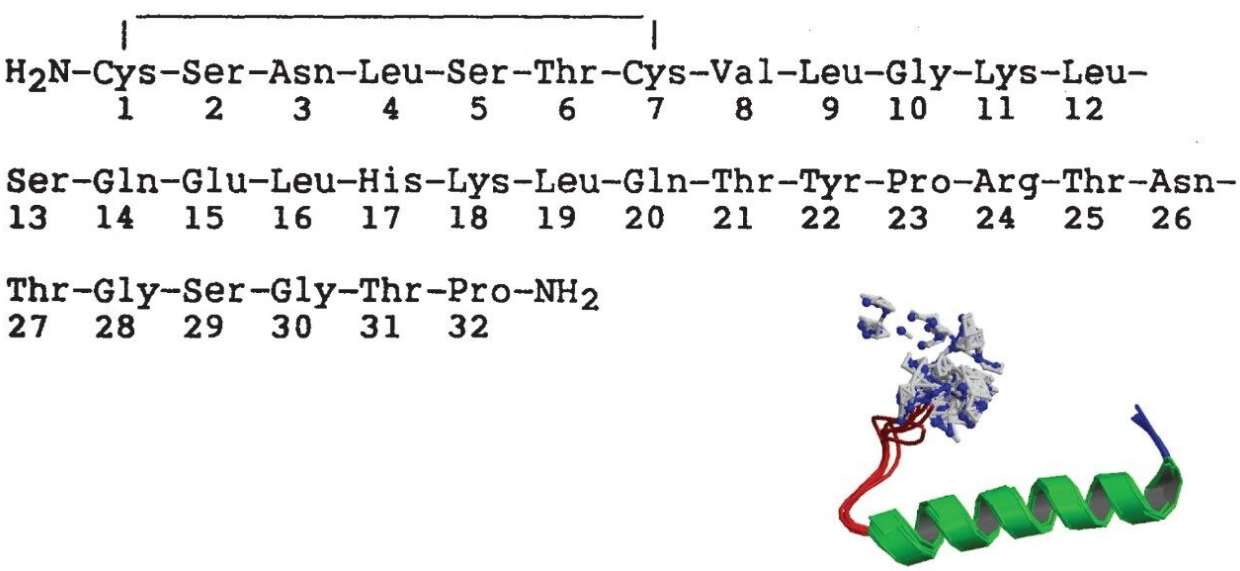

Figure 1. Salmon calcitonin (sCT) amino acid sequence and structure.

Experimental

Sample preparation

Preparation of samples, calibration standards, and QC samples

Calibration curve standards and quality control (QC) samples of sCT were prepared at various concentration levels (25–1,500 pg/mL) in commercially available human serum. All calibration curve standards, QC levels, and blank (non-spiked) serum samples were prepared in triplicate.

Protein precipitation (PPT)

100 µL of serum was precipitated with 100 µL acetonitrile, vortexed and centrifuged at 13,000 RCF for 10 minutes. A 150 µL aliquot of the supernatant was transferred into a 2 mL, 96-well sample collection plate (p/n: 186002482) containing 600 µL of 4% phosphoric acid and mixed.

SPE using Oasis WCX 96-well µElution Plate

The diluted PPT supernatant was loaded onto a 96-well Oasis WCX µElution Plate (p/n:186002499) in 2 ×

375 µL aliquots. All wells were subsequently washed with 200 µL of 5% ammonium hydroxide followed by 200 µL of 20% acetonitrile. Salmon calcitonin was then eluted from the sorbent using 1 × 25 µL aliquot of the elution solvent containing 1% trifluoroacetic acid in 75/25 (v/v) acetonitrile/water. All samples were then diluted with 25 µL water for a final sample volume of 50 µL. Total time required for sample extraction was <2 hours.

LC-MS conditions

LC system:	ACQUITY UPLC I-Class
Detection:	Xevo TQ-XS Tandem Quadrupole Mass Spectrometer, ESI+
Column:	CORTECS UPLC C_{18}+, 90Å, 1.6 µm, 2.1 × 50 mm (p/n: 176003167)
Temp.:	60 °C
Sample temp.:	5 °C
Injection volume:	20 µL
Mobile phases:	A: 0.1% Formic acid in H_2O B: 0.1% Formic acid in ACN

Gradient:

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	95	5	6
0.5	0.4	95	5	6

Time (min)	Flow rate (mL/min)	%A	%B	Curve
2.5	0.4	25	75	6
2.6	0.4	5	95	6
3.5	0.4	5	95	6
3.6	0.4	95	5	6
4.5	0.4	95	5	6

Precursor (*m/z*)	Product (*m/z*)	Cone voltage	Collision energy	
687.5	830.3	12	10	Primary
859.2	1106.7	20	18	Confirmatory

Table 1. Final MS conditions for salmon calcitonin, including precursor and fragment ions.

Results and Discussion

Mass Spectometry

LC-MS/MS quantification of sCT was performed using the Xevo TQ-XS Tandem Quadrupole Mass Spectrometer in ESI+ mode. During method development, several multiply charge precursors were observed (Figure 2). Of these, the 4+ (*m/z* 859.2) and 5+ (*m/z* 687.5) precursors were the most predominant species and yielded highly selective fragments which were used for quantification. The MRM transition using 687.5 → 830.3 was used as the primary quantification transition, while the MRM transition 859.2 → 1106.7 was used as a qualifier. Optimized MS conditions and MRM transitions used for the quantification of sCT are listed in

Table 1.

Although many peptides and proteins produce intense fragments below *m/z* 200, these ions (often immonium ions) result in high background in extracted samples due to their lack of specificity. In this assay, the use of highly specific b/y ion fragments above *m/z* 800 yielded significantly improved specificity, facilitating the use of simpler LC and SPE methodologies.

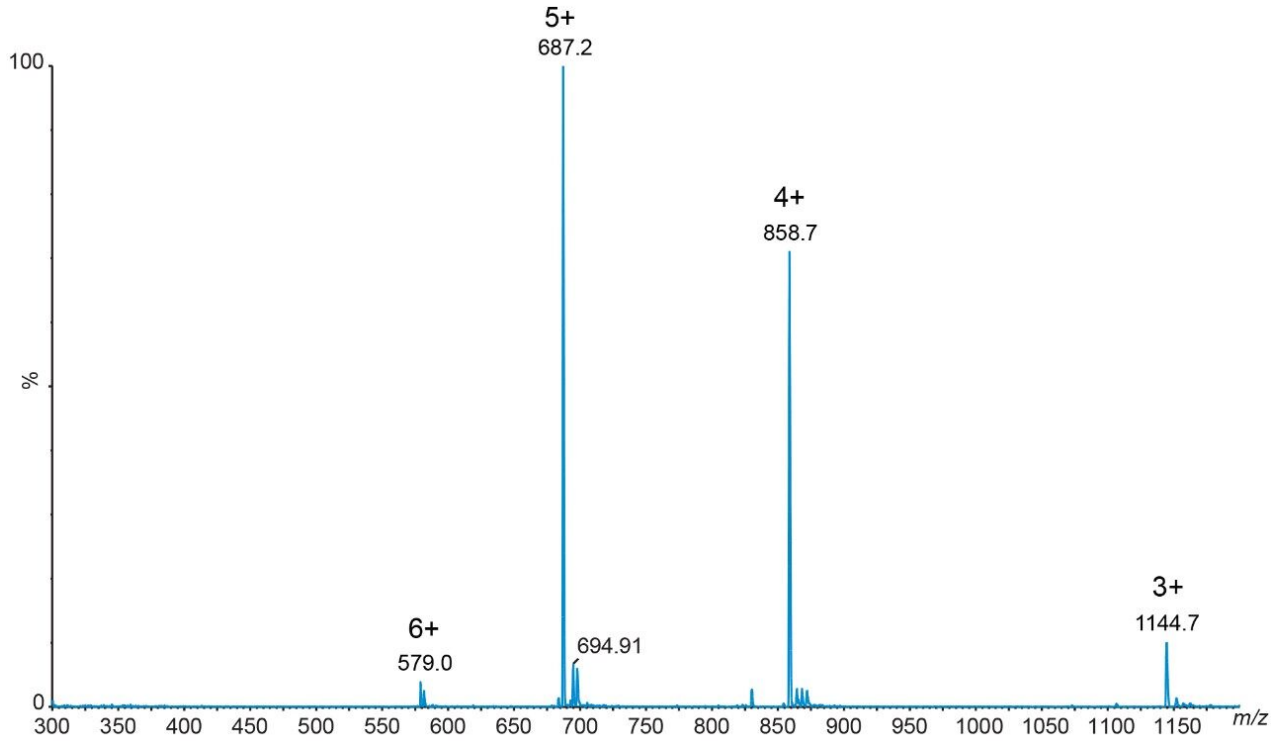

Figure 2. Representative MS scan spectra of salmon calcitonin highlighting the 4+ (m/z 859.2) and 5+ (m/z 687.5) precursors used for quantification.

Liquid Chromatography

During sCT method development, several reversed-phase columns: ACQUITY UPLC Peptide HSS T3, ACQUITY UPLC Peptide BEH C_{18}, ACQUITY UPLC Peptide CSH C_{18}, and CORTECS UPLC C_{18}+ were evaluated for overall chromatographic performance (e.g., assessment of peak shape, area counts, and signal to noise). Best chromatographic separation was achieved using an CORTECS UPLC C_{18}+, 90Å, 1.6 µm, 2.1 × 50 mm Column (p/n: 176003167) and 0.1% formic acid in water and acetonitrile. Unlike small molecules, larger peptides and small proteins, like sCT, suffer from poor mass transfer in and out of fully-porous particles. Thus, use of a sub-2-µm solid-core CORTECS UPLC C_{18}+ Column, with its low level positive surface charge, provided significantly narrower peak widths (<4 seconds) than the traditional C_{18} column

(>12 seconds) and resulted in 10-fold improvement in S/N (Figure 3).

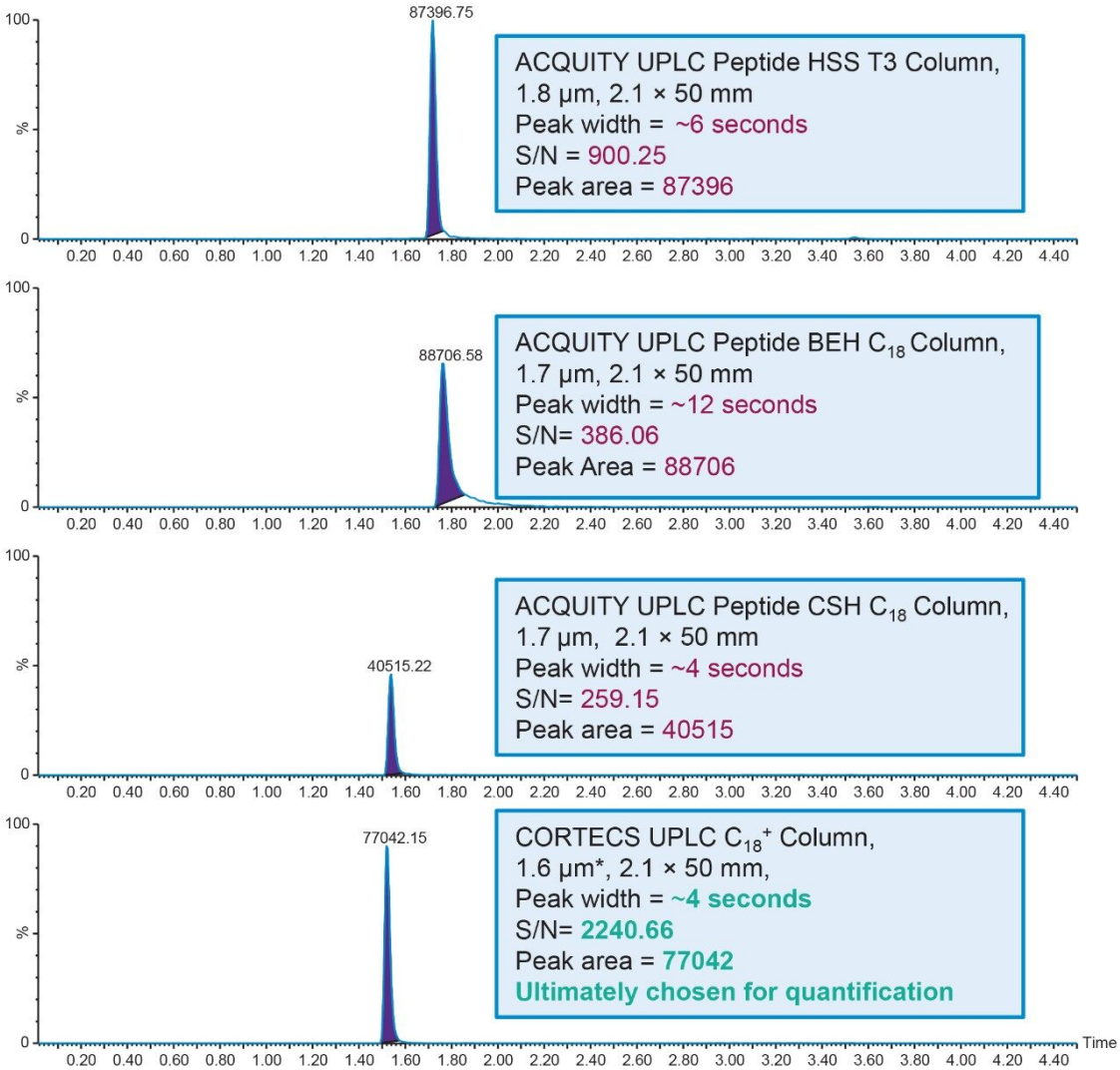

Figure 3. Column performance evaluation of the ACQUITY UPLC Peptide HSS T3, ACQUITY UPLC Peptide BEH C_{18}, ACQUITY UPLC Peptide CSH C_{18} , and CORTECS C_{18}+ Columns for salmon calcitonin. The CORTECS C_{18}+ Column provided best overall chromatographic performance with improvements in peak area, S/N, and peak widths (<4 seconds).

Sample Preparation

Development of sensitive and selective sample extraction strategies for peptides can often be challenging due to issues associated with non-specific binding (adsorption), protein binding, and difficulty maintaining solubility throughout the sample extraction process. While protein precipitation is a very common and effective sample preparation strategy for quantification of small molecules from serum/plasma (effectively

disrupting protein binding, affording high recovery, and minimizing matrix interferences), it is often detrimental for large molecule (peptide/protein) bioanalytical quantification. Due to their size and nature, protein precipitation, using high ratios of organic solvent, often results in peptide loss due to undesired precipitation of the peptide itself. For sCT, as the ratio of acetonitrile to serum increased, the recovery greatly decreased (Figure 4).

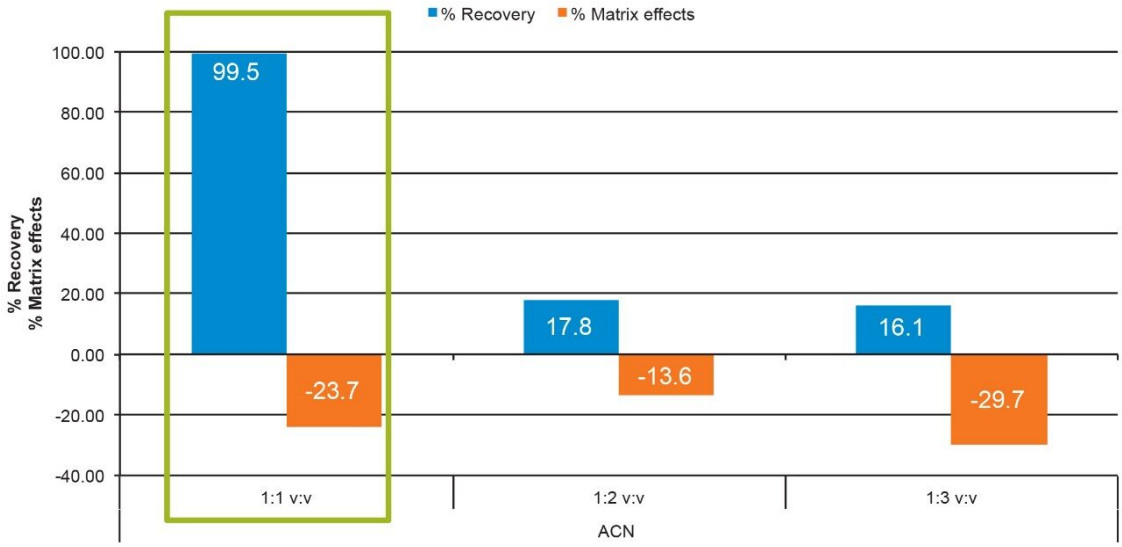

Figure 4. Salmon calcitonin protein precipitation (PPT) serum extraction recovery and matrix effects.

For peptides, solid-phase extraction (SPE) sample preparation is ideal. It provides rapid sample cleanup, high recovery, sample concentration without the need for sample evaporation, and helps ensure peptide solubility throughout the extraction process. Use of the Waters Peptide Separation Technologies (PST) µElution SPE screening protocol, highlighted in Figure 5, allows the user to quickly evaluate SPE sorbents and assess initial SPE recovery for sCT.

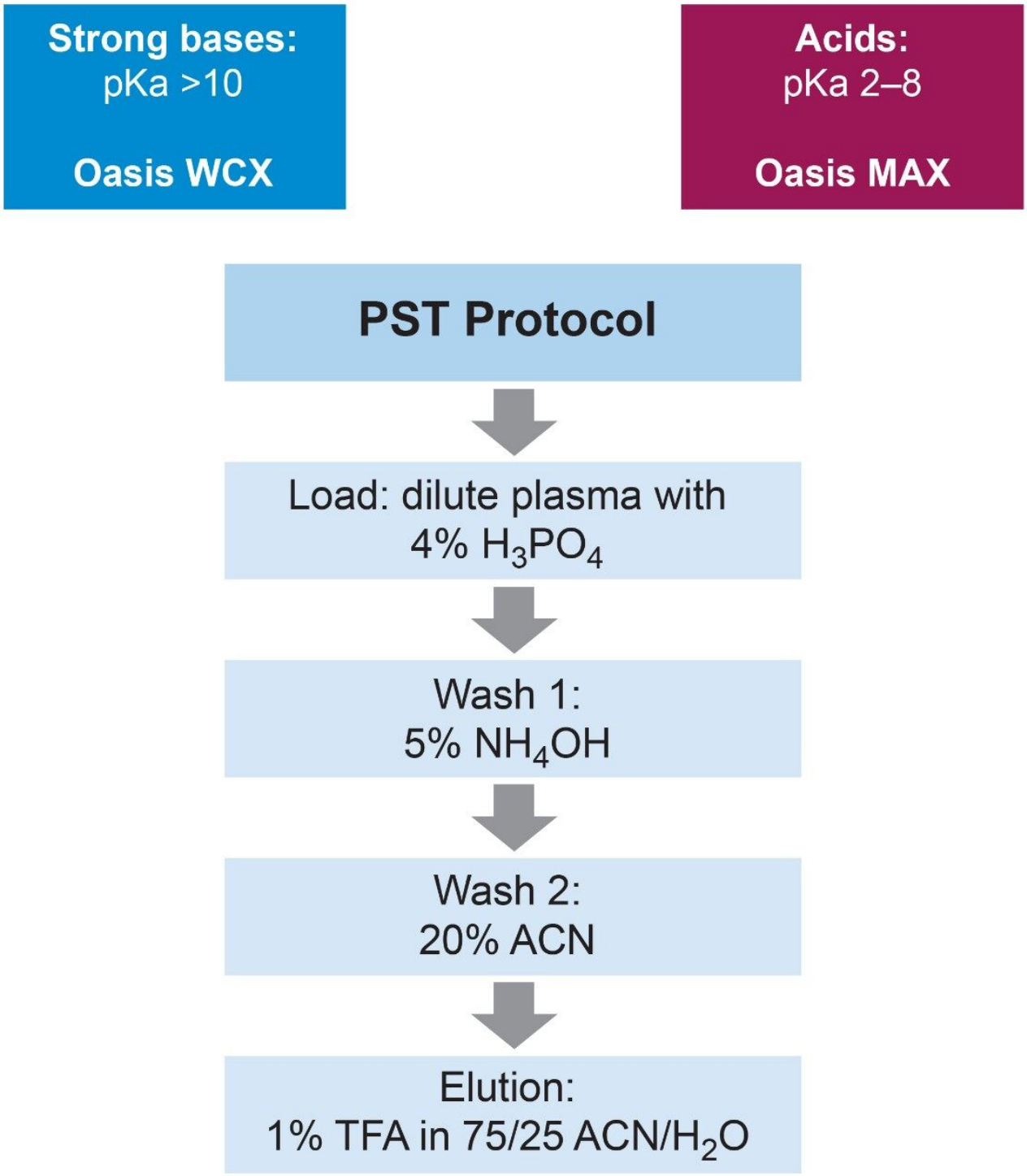

Figure 5. Waters Oasis PST SPE protocol for peptides.

Results from this assessment determined that the Oasis WCX sorbent yielded much better recovery vs. the Oasis MAX sorbent (60 vs. 4%) for sCT (Figure 6).

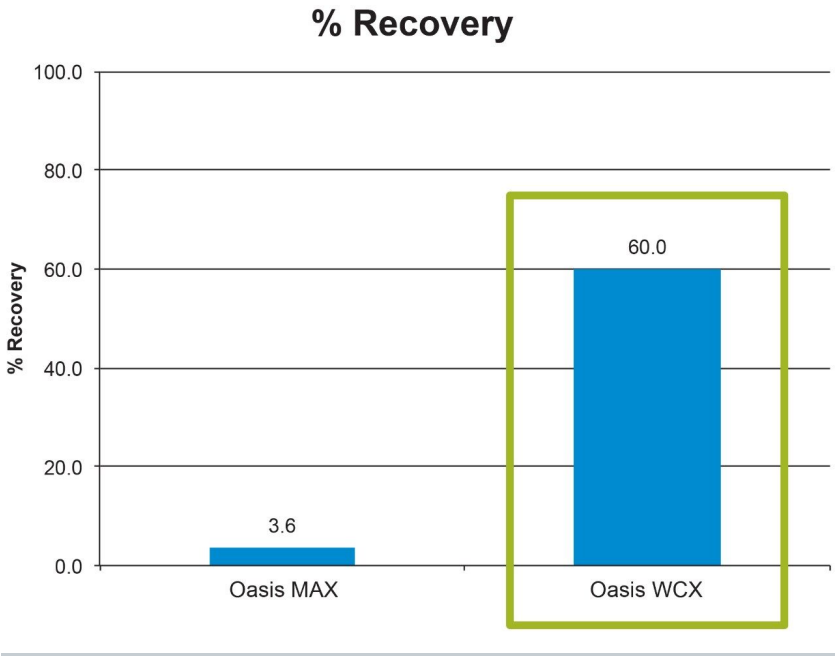

Figure 6. Extraction recovery results using Oasis MAX and WCX sorbents and PST SPE screening protocol.

Sample pretreatment prior to SPE proved to be critical for improving recovery and specificity. Using a 1:1 protein precipitation with acetonitrile resulted in ~100% recovery without precipitating the peptide itself. The PPT pretreatment minimized protein binding and eliminated endogenous interferences from large proteins such as albumin. Final sample pretreatment and SPE conditions are described in the experimental section and are illustrated in Figure 7.

Using this protein precipitation strategy in combination with µElution SPE, provided high recovery (>90%) and greatly reduced the matrix effects (<15%), improving overall specificity and sensitivity for sCT.

This performance is highlighted in Figure 8. In addition, due to the water wettable nature of the Oasis sorbents, we were able to eliminate the conditioning and equilibration steps for this method, reducing time and number of steps.

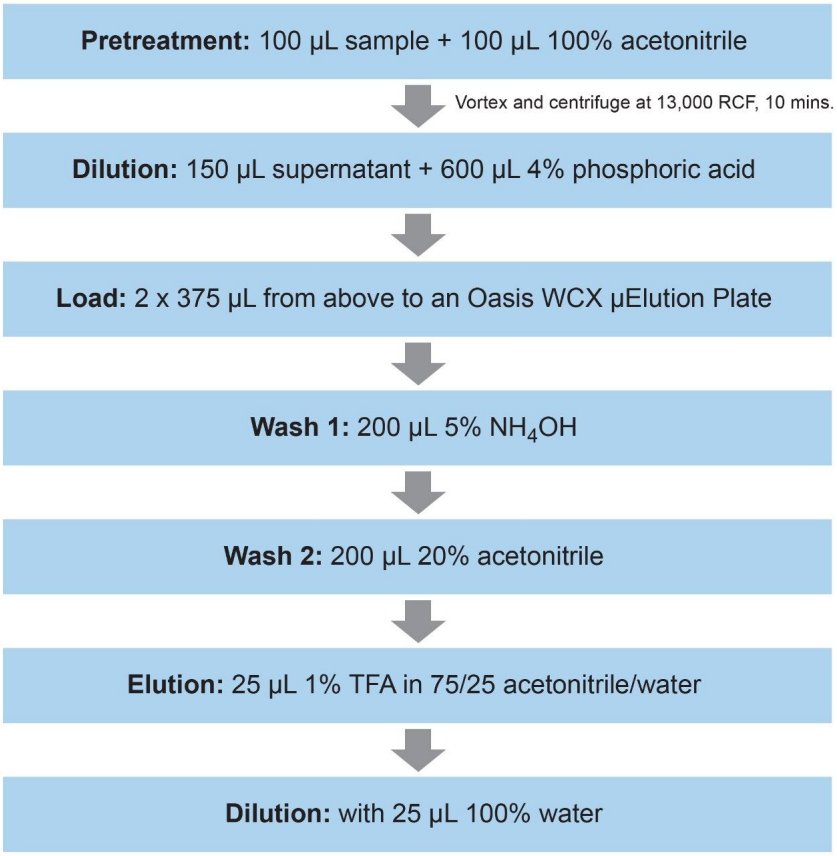

Figure 7. Final extraction protocol for salmon calcitonin using Oasis WCX 96-well µElution Plate.

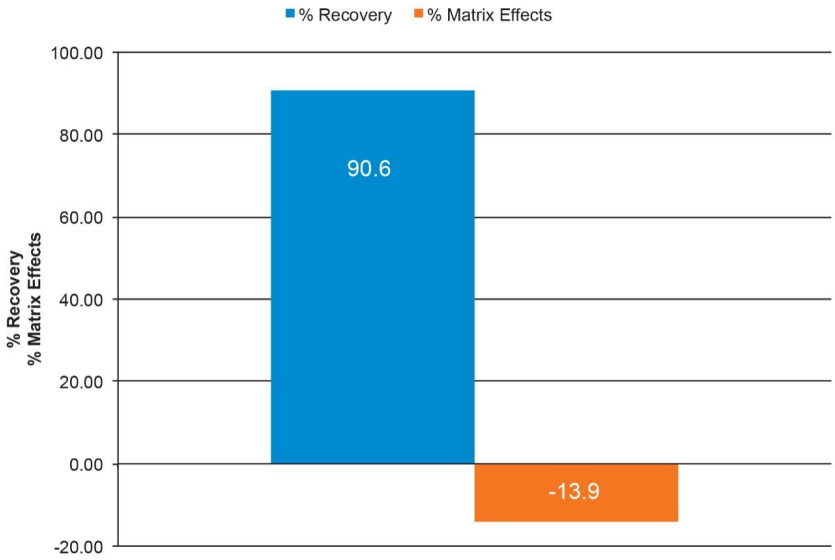

Figure 8. Salmon calcitonin serum extraction recovery results using the optimized SPE protocol with PPT sample pretreatment and mixed-mode (Oasis WCX) SPE in the 96-well µElution Plate format.

Linearity, Accuracy and Precision

Using only 100 µL of human serum and the selective sample cleanup method described above, this method achieved a LLOQ of 25 pg/mL for sCT. Using a 1/x linear fit, calibration curves were linear (r^2 >0.99) from 25–1,500 pg/mL with accuracies between 85–115% and CVs <15% for all points on the curve. Additionally, both intra-day and inter-day accuracy and precision met recommended bioanalytical method validation guidelines. This QC performance is highlighted in Table 2, panels A (intra-day QC accuracy) and B (inter-day QC accuracy), while chromatographic performance is illustrated in Figure 9.

Intra-day precision and accuracy					
	Expected concentration (ng/mL)	Average observed concentration (pg/mL) N=3	Standard deviation	% Accuracy	% CV
LQC	75	77.20	9.87	97.07	12.79
MQC	250	240.63	21.27	103.75	8.84
HQC	1000	891.70	82.36	110.83	9.24

Inter-day precision and accuracy					
	Expected concentration (ng/mL)	Average observed concentration (pg/mL) N=9	Standard deviation	% Accuracy	% CV
LQC	75	75.71	8.49	99.05	11.22
MQC	250	242.21	20.15	103.12	8.32
HQC	1000	993.66	108.35	100.63	10.90

Table 2. Intra (panel A) and inter-day (panel B) QC quantitative performance for salmon calcitonin.

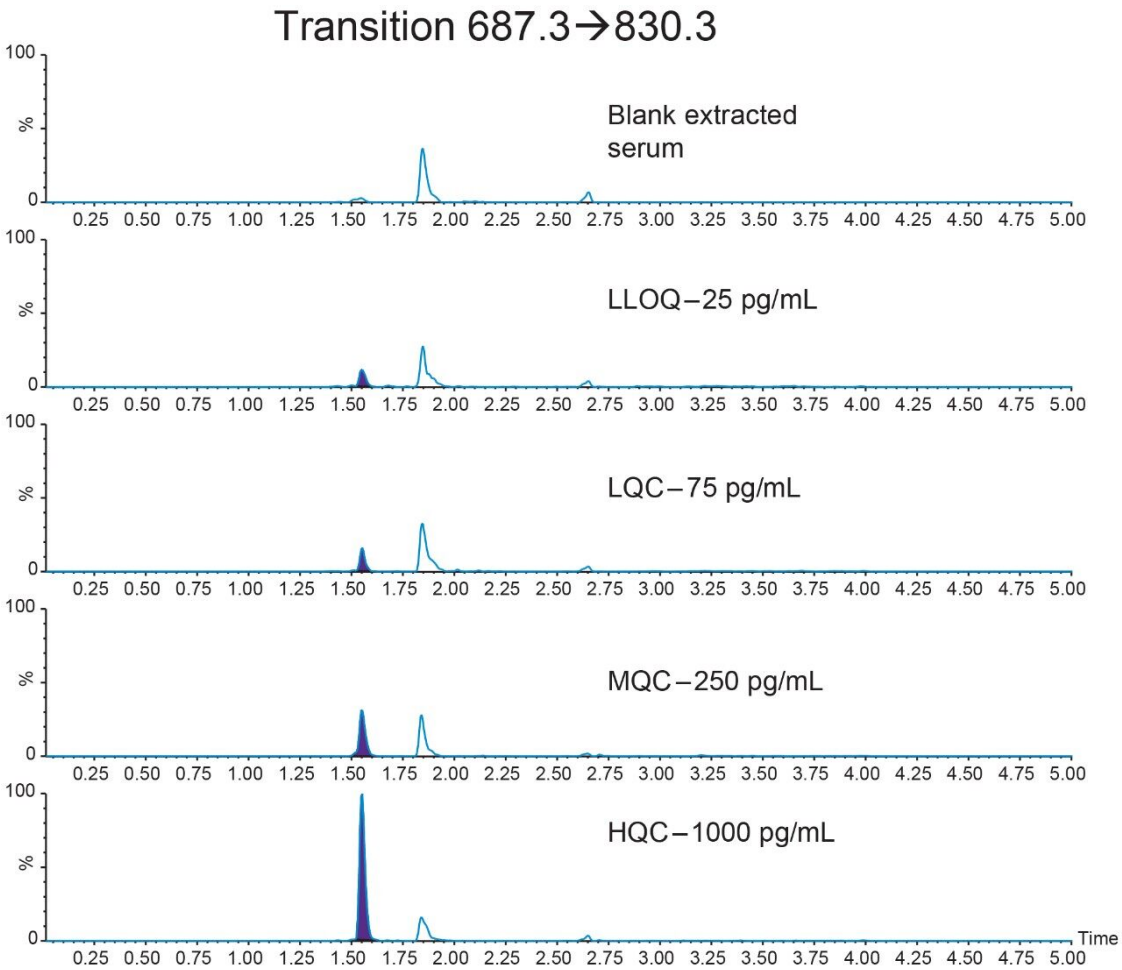

Figure 9. Representative QC chromatograms for salmon calcitonin extracted using WCX µElution SPE from 100 µL of serum.

Conclusion

This method describes a simple sample preparation strategy using Oasis WCX µElution SPE combined with analytical flow LC and a tandem (triple) quadrupole MS for the high sensitivity quantification of salmon calcitonin from human serum.

- Sample preparation with simple SPE eliminated the need for complex sample preparation with protein digestion or affinity chromatography.
- Use of a solid-core, sub-2-µm CORTECS UPLC C_{18}+ Column provided a fast, simple LC method with an

analysis time of 5 minutes.

- Using only 100 µL of sample, a LLOQ of 25 pg/mL was achieved without altering the standard resolution of a triple quadrupole instrument.
- The excellent quantitative performance of the method described, reliably measures low levels of salmon calcitonin from serum.

References

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720006342, July 2018

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