Challenging Matrix: How to extract a Peptide-Analogue from Human Breast Milk for LC-MS/MS analysis?

Sergio Menta, Martin Köhler, Werner Meyer, Matthias Sury and Petra Struwe
Celerion Switzerland AG, 8320 Fehraltorf, Switzerland

INTRODUCTION

In clinical development the excretion of a peptide-analogue into breast milk should be investigated to evaluate the safety of the medication during breastfeeding.

However, the extraction of biologically active molecules from human breast milk is very challenging, due to the complexity and heterogeneity of this biological fluid, mainly composed of proteins, lipids, sugars and minerals.

For LC-MS/MS based analytical techniques, specific extraction methods need to be developed to optimize analyte recovery from breast milk, and to avoid ionization suppression, high background noise, interferences and column overpressure. Thus, protocols optimized for plasma cannot be directly translated to milk.

Here, we present a fast and highly effective automated extraction method for a peptide-analogue in human breast milk, based on organic solvent protein precipitation followed by liquid-liquid extraction. Combining the two extraction steps proved to be efficient in removing breast milk proteins and lipids, resulting in a robust and selective bioanalytical method, which was fully validated according to FDA and EMA regulatory guidelines in the analytical range of 3.00 – 250 ng/mL and has been proven to be suitable for clinical sample analysis.

SAMPLE EXTRACTION PROCEDURE

Sample transfer
- 100 µL of breast milk sample was transferred into a 96-deep well plate
- 20 µL of internal standard solution was added

Protein Precipitation
- Addition of 500 µL ice cold methanol to all samples
- Centrifugation at 1500 xg for 15 min
- Supernatant was transferred using Hamilton Microlab Starlet 96-Channel robotic system

Liquid-liquid Extraction
- Addition of 400 µL chloroform
- Addition of Millipore water
- Vortexing followed by centrifugation
- Aqueous biphasic system: upper phase contains hydrophilic compounds (e.g. peptides) and lower phase hydrophobic compounds (e.g. lipids)
- Upper phase was transferred using Hamilton Microlab Starlet 96-Channel robotic system

Evaporation Reconstitution
- Evaporation of transferred upper phase
- Reconstitution in Millipore water + 0.1 % formic acid

Figure 1: Sample extraction scheme for a peptide-analogue in human breast milk. Additional organic solvents were tested for liquid-liquid extraction instead of chloroform: (1) dichloromethane showed low recovery and (2) hexane showed high background noise and low signal intensity. Furthermore, direct LC injection of upper phase showed high background noise and therefore evaporation and reconstitution is needed.

LC-MS/MS CONDITIONS

Chromatographic conditions	
UHPLC	Waters ACQUITY UPLC I-Class
Analytical column	Waters ACQUITY UPLC BEH C18, 300Å, 50 x 2.1 mm, 1.7 µm
Mobile phase A **Mobile phase B**	Millipore water / Formic acid (100:1 (v/v)) Millipore water / Acetonitrile / Methanol / Formic acid (5:85:15:1 (v/v/v/v))
Flow rate **Column temperature** **Injection volume**	0.9 mL/min 30 °C 20 µL
Total run time	5.0 min
MS/MS conditions	
Mass spectrometer	Triple Quad 6500+
Source/Polarity	MRM, Positive

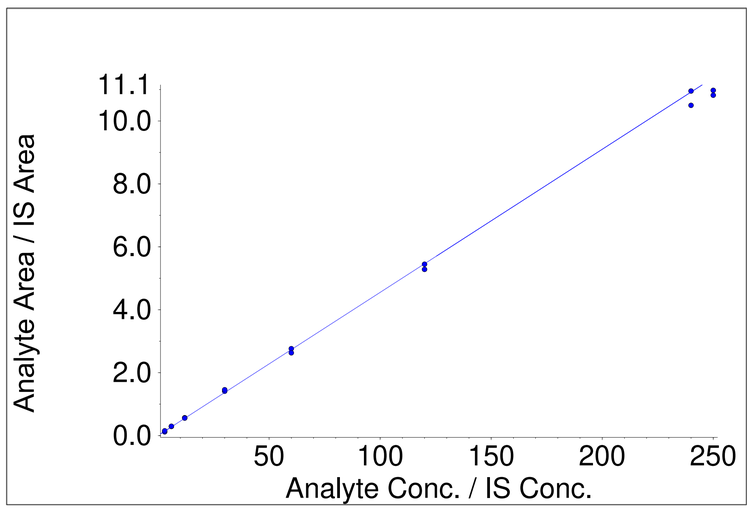

Figure 2: Calibration curve covering a range of 3.00 – 250 ng/mL with a goodness-of-fit measure of R2 = 0.998

CHROMATOGRAMS

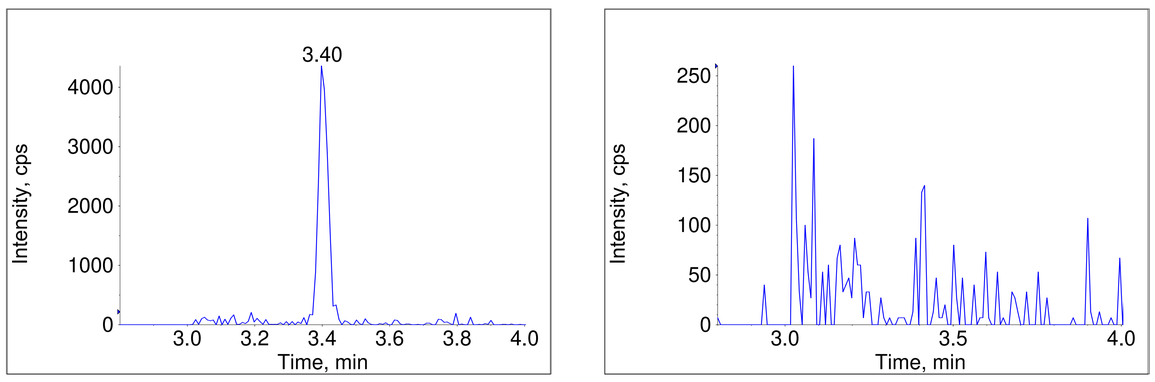

Figure 3: Chromatogram of the LLOQ QC (left) showed well a resolved analyte signal (S/N >160). In blank samples (right) low background noise was detected illustrating the efficient removal of matrix compounds.

RESULTS

Within-Batch and Between-Batch Precision and Accuracy Results		QC LLOQ 3.00 ng/mL	QC Low 9.00 ng/mL	QC Med 40.0 ng/mL	QC High 200 ng/mL	DQC* 1000 ng/mL
Run 1	**Accuracy (%)** **CV (%)** **n**	100.1 7.5 6	104.6 3.9 6	102.3 2.6 6	98.4 2.4 6	94.7 1.7 6
Run 2	**Accuracy (%)** **CV (%)** **n**	96.8 10.2 6	104.6 1.6 6	103.8 2.9 6	99.0 1.5 6	
Run 3	**Accuracy (%)** **CV (%)** **n**	98.1 16.3 6	100.5 5.9 6	103.8 4.5 6	99.0 2.8 6	
Inter-batch Precision and Accuracy	**Accuracy (%)** **CV (%)** **n**	**99.6** **11.4** **18**	**103.2** **4.3** **18**	**102.8** **3.3** **18**	**98.8** **2.2** **18**	

*DQC was 5-fold diluted using a pool of human breast milk

Matrix Effect Results	Matrix ID	ME Low 9.00 ng/mL	% Bias	ME High 200 ng/mL	% Bias
Run 7	ME1	8.92	-0.9	192	-3.9
	ME2	8.79	-2.3	203	1.6
	ME3	9.12	1.4	208	4.1
	ME4	8.84	-1.8	193	-3.5
	ME5	9.16	1.8	206	3.1
	ME6	9.41	4.6	212	6.2
	ME7	9.21	2.3	207	3.3
Mean (ng/mL) **CV (%)** **N**		**9.06** **2.5** **7**		**203** **3.8** **7**	

In addition, Selectivity, Carryover, Matrix Effect, and Stabilities in matrix were evaluated and acceptance criteria were met.