Supplementary information for:

High-Sensitivity LC-MS/MS Quantification of Peptides and Proteins in Complex Biological Samples: the Impact of Enzymatic Digestion and Internal Standard Selection on Method Performance.

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Total protein assay

The total protein content of samples was determined with a total protein assay, based on bicinchoninic acid (BCA) used according to the manufacturer's instructions, Pierce ® BCA Protein Assay Kit , product number 23225

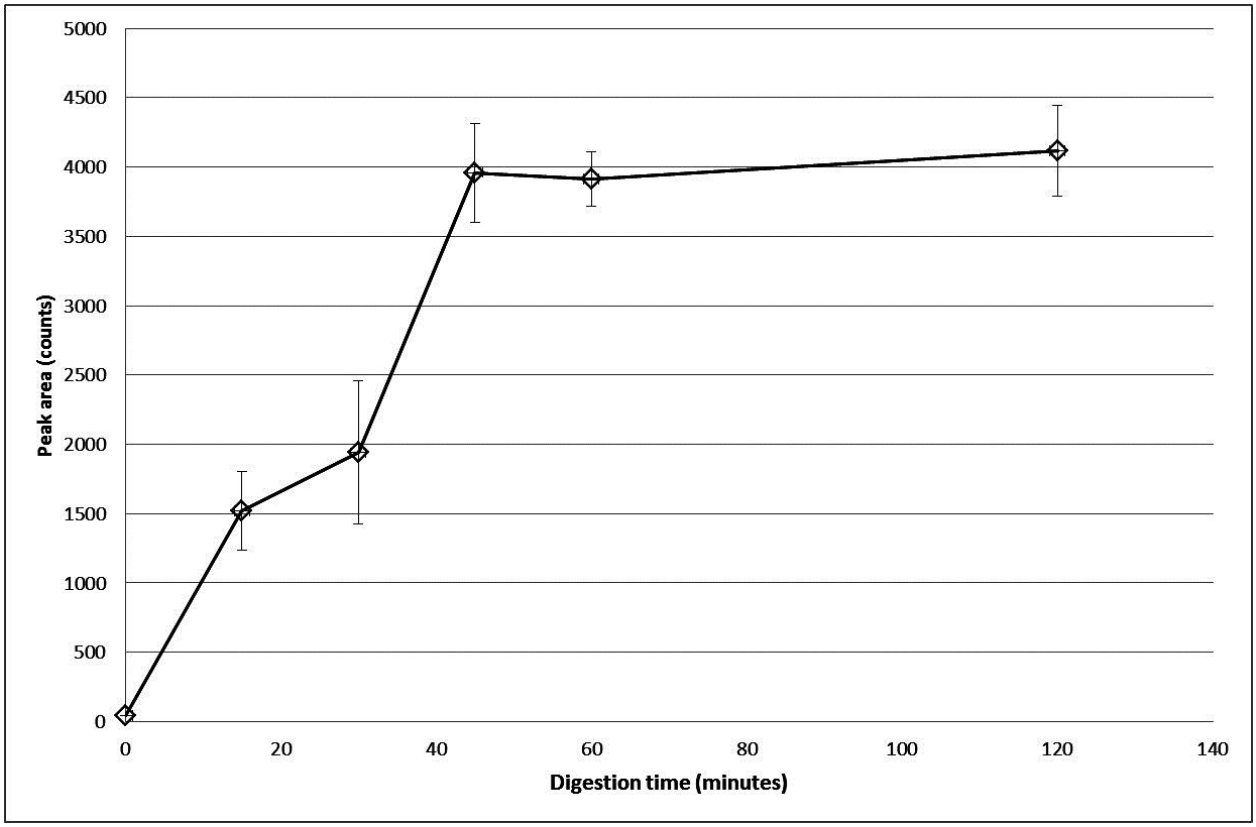

Figure S1. Digestion time course

The time course of the trypsin digestion of salmon calcitonin after solid phase extraction from plasma. After one hour, a plateau was reached, which indicates the digestion has reached completeness. however to account for experimental variability, a two hour digestion time period was used.

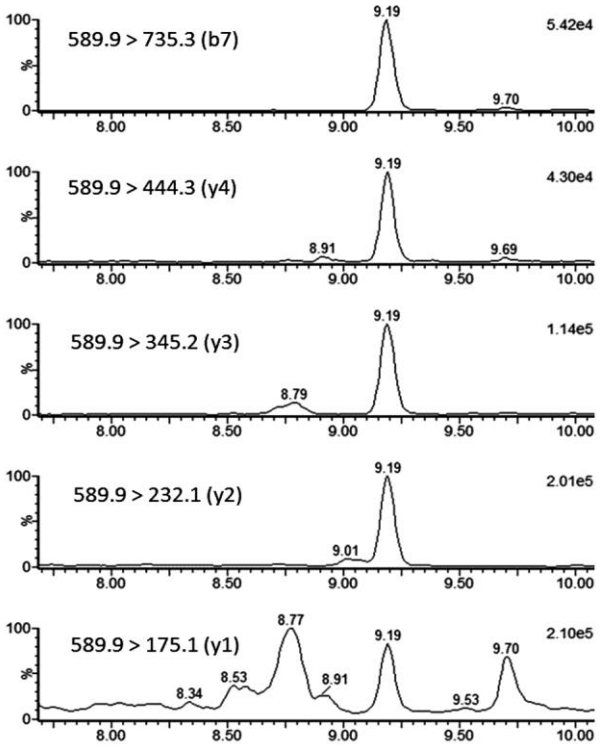

Figure S2 SRM transition selectivity.

LC-MS/MS chromatograms of five different SRM transitions for the di-methylated signature peptide [1-11] of salmon calcitonin. Mass transitions (left hand side) as well as signal intensities (right hand side) are indicated in the chromatograms

More interferences appear in the chromatograms for smaller fragment ions, most probably because they contain less specific molecular information and can more easily be formed by fragmentation of other peptides. Despite the lower selectivity for smaller fragment ions, they were in general, more intense.

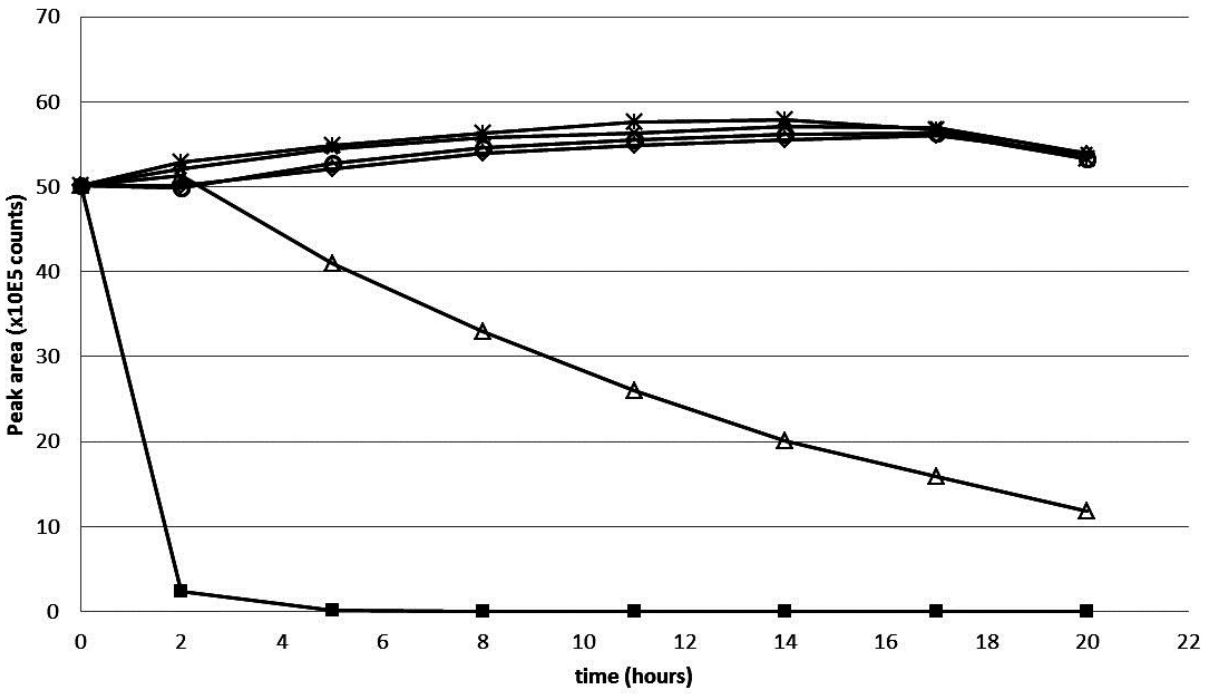

Figure S3. Back-exchange prevention approaches

Several approaches to prevent ^{18}O back-exchange were tested on digested plasma extracts: trypsin inhibition by the addition of 5% v/v of formic acid (—△—), boiling at 100°C for 10 minutes (—✳—), or the addition of an equimolar (—✳—) or double amount (—⊖—) of soybean trypsin inhibitor. These treatments were compared to the situation in which no trypsin deactivation was performed (—■—), as well as to the situation where no trypsin had been added (—◇—). The ^{18}O-labeled internal standard was added to the samples after these treatments, and the samples were subsequently stored in the autosampler and regularly injected during 21 hours.

The results demonstrate that by boiling the samples or by the addition at least an equimolar amount of soybean trypsin inhibitor, the trypsin activity can be reduced in a way that stabilizes the ^{18}O-labeled internal standard in the autosampler for at least 21 hours. In the final work-flow, a double amount of soybean trypsin inhibitor was added to the digest.

level	analytical method		
(pg/ml)	no digestion	digestion	digestion and derivatization
10	cal + QC + M	-	-
20	cal + QC	-	-
50	cal	cal + QC + M	-
100	cal + QC	cal + QC	cal + QC + M
200	cal	cal	cal + QC
500	cal	cal	cal
1000	cal + QC	cal + QC	cal + QC
2000	-	cal	cal
5000	-	cal + QC	cal
10000	-	-	cal+ QC

Table S1 Preparation of plasma samples

Plasma samples were prepared at different levels for each work-flow, allowing for the observed differences in sensitivity. The different workflows (no digestion, digestion and digestion and derivatization) are shown with their respective calibration (cal), quality-control (QC), and matrix variability (M) levels. The QC-samples prepared at 100 pg/mL and 1000 pg/mL were compared for each work-flow.

Name	Q1		Q3		cone	CV	DT	CE
	ion	m/z	ion	m/z	(V)	(kV)	(°C)	(V)
Salmon calcitonin [1-32]	$[M+4H]^{4+}$	858.4	$(b31)^{3+}$	1106.7	40	3	400	20
Human calcitonin [1-32]	$[M+3H]^{3+}$	1141.1	$(y1)^{1+}$	115.1	40	3	400	25
SIL-salmon calcitonin [1-32]	$[M+4H]^{4+}$	860.4	$(b31)^{3+}$	1108.9	40	3	400	20
Eel calcitonin [1-32]	$[M+4H]^{4+}$	854.2	$(b31)^{3+}$	1100.6	40	3	400	20
Salmon calcitonin [1-11]	$[M+2H]^{2+}$	561.9	$(y2)^{1+}$	204.0	40	3	400	20
Human calcitonin [19-32]	$[M+2H]^{2+}$	721.5	$(y1)^{1+}$	115.1	40	3	400	20
SIL-salmon calcitonin [1-11] [a)]	$[M+2H]^{2+}$	565.8	$(y2)^{1+}$	212.1	40	3	400	25
^{18}O-salmon calcitonin [1-11]	$[M+2H]^{2+}$	563.9	$(y2)^{1+}$	208.0	40	3	400	20
Derivatized salmon calcitonin [1-11]	$[M+2H]^{2+}$	589.9	$(b7)^{1+}$	735.3	50	3	400	25
Diff. derivatized salmon calcitonin [1-11]	$[M+2H]^{2+}$	593.9	$(b7)^{1+}$	739.3	50	3	400	25

Table S2. MS settings used

For all monitored compounds, the detected molecular ion (Q1 ion) and its corresponding mass to charge ratio (m/z), as well as its corresponding fragment ion (Q3 ion) and mass to charge ratio are given. More detection settings: cone voltage (cone), Capillary voltage (CV), desolvation temperature (DT) and collision energy (CE).

	M-var			QC-LLOQ			QC-low		
Level (pg/ml)	10.0			10.0			20.0		
IS Approach:	Avg.	CV	Bias	Avg.	CV	Bias	Avg.	CV	Bias
No internal standard	8.9	14.3	-10.7	8.8	18.7	-12.3	18.8	15.1	-5.9
SIL-salmon calcitonin	9.7	9.0	-3.2	9.9	7.0	-0.7	17.9	12.6	-10.7
Eel calcitonin	8.4	19.1	-15.7	12.2	41.2	21.5	17.9	11.3	-10.3
Human calcitonin	7.7	43.1	-23.3	7.6	27.5	-23.7	18.8	30.7	-6.0

	QC-medium			QC-high		
Level (pg/ml)	100			1000		
IS Approach:	Avg.	CV	Bias	Avg.	CV	Bias
no internal standard	117.4	7.1	17.4	1096.3	9.4	9.6
SIL-salmon calcitonin	103.7	3.5	3.7	989.8	1.8	-1.0
eel calcitonin	120.4	31.1	20.4	1025.6	9.0	2.6
human calcitonin	171.1	18.8	71.1	1588.6	19.7	58.9

Table S3. Accuracy and precision results for work-flow A.

For work-flow A, spiked plasma samples were analyzed in six-fold and their responses calculated for each internal standardization approach. The obtained average value (Avg.), the coefficient of variation (CV) and the deviation from the nominal value (Bias) are shown.

	M-var			QC-LLOQ			QC-low		
Level (pg/ml)	50.0			50.0			100.0		
IS Approach:	Avg.	CV	Bias	Avg.	CV	Bias	Avg.	CV	Bias
no internal standard	51.0	2.2	1.9	48.9	8.4	-2.2	101	6.1	0.9
SIL-salmon calc. [1-32]	49.3	3.6	-1.4	50.1	2.6	0.1	102	5.1	1.5
Cleav. SIL peptide	53.0	2.4	5.9	53.6	2.9	7.1	110	6.2	10.2
Human calc. [1-32]	43.0	9.1	-14.0	48.4	6.6	-3.1	79.8	8.3	20.2

	QC-medium			QC-high		
Level (pg/ml)	1000			5000		
IS Approach:	Avg.	CV	Bias	Avg.	CV	Bias
no internal standard	1072	3.6	7.2	5596	1.6	11.9
SIL-salmon calc. [1-32]	1010	3.2	1.0	5105	3.0	2.1
Cleav. SIL peptide	1044	2.9	4.4	5672	2.2	13.4
Human calc. [1-32]	888.4	1.9	-11.2	4665	2.6	-6.7

Table S4. Accuracy and precision results for work-flow B-before

For work-flow B-before, in which the internal standards were added to the samples before the digestion step, and subsequently co-digested, plasma samples were analyzed in six-fold and their responses calculated for each internal standardization approach. The obtained average value (Avg.), the coefficient of variation (CV) and the deviation from the nominal value (Bias) are shown.

	M-var			QC-LLOQ			QC-low		
Level (pg/ml)	50.0			50.0			100.0		
IS Approach:	Avg.	CV	Bias	Avg.	CV	Bias	Avg.	CV	Bias
SIL-peptide [1-11]	53.0	2.4	5.9	53.6	2.9	7.1	110	6.2	10.2
Human calc. [1-32]	53.5	41.1	6.9	57.9	14.3	15.7	152	19.0	51.6
^{18}O-exchange [1-11]	47.5	4.0	-5.0	46.2	5.4	-7.6	93.7	2.6	-6.3

	QC-medium			QC-high		
Level (pg/ml)	1000			5000		
IS Approach:	Avg.	CV	Bias	Avg.	CV	Bias
SIL-peptide [1-11]	1044	2.9	4.4	5535	2.1	10.7
Human calc. [1-32]	959.1	17.0	-4.1	5555	53.6	11.1
^{18}O-exchange [1-11]	1003	4.1	0.3	5226	4.5	4.5

Table S5. Accuracy and precision results for work-flow B-after

For work-flow B-after, in which the internal standards were added to the samples following the digestion step, plasma samples were analyzed in six-fold and their responses calculated for each internal standardization approach. The obtained average value (Avg.), the coefficient of variation (CV) and the deviation from the nominal value (Bias) are shown.

	M-var			QC-LLOQ			QC-low		
Level (pg/ml)	100			100			200		
IS Approach:	Avg.	CV	Bias	Avg.	CV	Bias	Avg.	CV	Bias
no internal standard	88.2	15.0	-11.8	101	7.0	0.7	228	3.2	14.2
differential deriv.	89.2	7.4	-10.8	94.1	9.3	-5.9	225	5.9	12.3

	QC-medium			QC-high		
Level (pg/ml)	1000			10000		
IS Approach:	Avg.	CV	Bias	Avg.	CV	Bias
no internal standard	829	3.0	-17.1	10707	3.8	7.1
differential deriv.	892	4.5	-10.8	10589	6.2	5.9

Table S6. Accuracy and precision results for work-flow C

For work-flow C, in which the internal standards was created by differential derivatization, plasma samples were analyzed in six-fold and their responses calculated for each internal standardization approach. The obtained average value (Avg.), the coefficient of variation (CV) and the deviation from the nominal value (Bias) are shown.