***Supplemental Data***

# Peptide Production and Decay Rates Affect the Quantitative Accuracy of Protein Cleavage Isotope Dilution Mass Spectrometry (PC-IDMS)

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*Molecular & Cellular Proteomics*
*2012*

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**Summary:** Herein are supporting information to the article titled above. The contents include additional details on: 1) the SRM transitions monitored for the specific detection of all native and SIL peptides 2) the derivation of the integrated rate equation used to model the measured peptide ratio, 3) the equations utilized for non-linear regression of experimental data, 4) the predictability of the mathematical model, 5) the derivation of the relative/percent error equation, 6) the predicted bias occurring when a SIL peptide standard is released via proteolysis, and 7) the dynamic range of protein production and decay rates observed for the targeted set of peptides.

## Supplemental Experimental Procedures

### SRM Transitions

All transitions monitored during these experiments are provided along with the collision energies (CE) employed to induce fragmentation (**Table S1**). Similar to previous reports (1, 2), the abundance of each transition was measured relative to all other transitions monitored for the same peptide species and compared to the same value measured using pure synthetic standards. Any transition observed to deviate significantly from the observations made with pure standards were excluded from use in quantification along with its corresponding isotope analogue. In some cases, transitions were also excluded from use in quantification due to observed isotope effects in the fragmentation efficiency.

TABLE SI
SRM Transitions Monitored for Each Target Peptide
Shown are the specific transitions and their respective collision energies used for each peptide investigated

| Peptide[a] | Ion Type | | NAT Ion m/z | | SIL Ion m/z | | CE (V)[b] |
|---|---|---|---|---|---|---|---|
| | Precursor | Product | Precursor | Product | Precursor | Product | |
| 4CL3.262-273 | $[M+2H^+]^{2+}$ | $y9^+$ | 659.884 | 943.582 | 663.393 | 950.599 | 23 |
| FDIGTLLGLIEK | | $y7^+$ | | 785.513 | | 792.530 | |
| | | $y6^+$ | | 672.429 | | 679.446 | |
| | | $y5^+$ | | 559.345 | | 566.362 | |
| | | $b2^+$ | | 263.103 | | 263.103 | |
| 4CL5.262-273 | $[M+2H^+]^{2+}$ | $y10^+$ | 659.885 | 1042.653 | 663.394 | 1049.670 | 23 |
| FEIGSLLGLIEK | | $y9^+$ | | 929.569 | | 936.586 | |
| | | $y6^+$ | | 672.431 | | 679.448 | |
| | | $y5^+$ | | 559.347 | | 566.364 | |
| | | $b2^+$ | | 277.118 | | 277.118 | |
| C3H3.125-134 | $[M+2H^+]^{2+}$ | $y8^+$ | 597.315 | 934.524 | 600.824 | 941.542 | 19 |
| VCTLELFSPK | | $y7^+$ | | 833.477 | | 840.494 | |
| | | $y6^+$ | | 720.393 | | 727.410 | |
| | | $y3^+$ | | 331.198 | | 331.198 | |
| C4H1.255-261 | $[M+2H^+]^{2+}$ | $y5^+$ | 472.211 | 665.325 | 475.218 | 671.339 | 15 |
| DYFVDER | | $y4^+$ | | 518.257 | | 524.271 | |
| | | $y3^+$ | | 419.188 | | 419.188 | |
| | | $b2^+$ | | 279.098 | | 279.098 | |
| | | $a2^+$ | | 251.103 | | 251.103 | |
| C4H2.255-261 | $[M+2H^+]^{2+}$ | $y5^+$ | 479.219 | 679.341 | 482.226 | 685.355 | 16 |
| DYFVEER | | $y4^+$ | | 532.273 | | 538.286 | |
| | | $y3^+$ | | 433.204 | | 433.204 | |
| | | $a2^+$ | | 251.103 | | 251.103 | |
| CAD1.184-198 | $[M+3H^+]^{3+}$ | $y12^{++}$ | 451.255 | 562.816 | 453.260 | 565.823 | 15 |
| GGILGLGGVGHMGVK | | $y11^{++}$ | | 506.274 | | 509.281 | |
| | | $y9^{++}$ | | 421.221 | | 424.228 | |

TABLE SI - continued

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| CAD2.177-185 | $[M+2H^+]^{2+}$ | $y8^+$ | 513.251 | 862.431 | 516.759 | 869.448 | 12 |
| YFGLDEPGK | | $y7^+$ | | 715.362 | | 722.379 | |
| | | $y5^+$ | | 545.257 | | 545.257 | |
| | | $y3^+$ | | 301.187 | | 301.187 | |
| | | $b2^+$ | | 311.139 | | 311.139 | |
| | | $a2^+$ | | 283.144 | | 283.144 | |
| CAD2.186-201 | $[M+3H^+]^{3+}$ | $y9^+$ | 509.644 | 837.494 | 511.983 | 844.511 | 21 |
| HIGIVGLGGLGHVAVK | | $y6^+$ | | 610.367 | | 610.367 | |
| | | $y9^{++}$ | | 419.251 | | 422.759 | |
| | | $b6^+$ | | 577.346 | | 577.346 | |
| | | b9+ | | 804.473 | | 804.473 | |
| CAld5H1.426-435 | $[M+2H^+]^{2+}$ | $y8^+$ | 574.803 | 888.446 | 577.810 | 894.460 | 18 |
| FLEPGVPDFK | | $y7^+$ | | 759.404 | | 765.417 | |
| | | $y4^+$ | | 506.261 | | 506.261 | |
| | | $b2^+$ | | 261.160 | | 261.160 | |
| | | $b3^+$ | | 390.202 | | 390.202 | |
| CAld5H2.427-436 | $[M+3H^+]^{3+}$ | $y4^+$ | 389.207 | 506.261 | 391.212 | 506.261 | 14 |
| FMKPGVPDFK | | $y2^+$ | | 294.181 | | 294.181 | |
| | | $y4^{++}$ | | 253.634 | | 253.634 | |
| | | $b6^+$ | | 660.354 | | 666.368 | |
| | | $a6^{++}$ | | 316.683 | | 319.690 | |
| CAld5H2.L.427-436 | $[M+3H^+]^{3+}$ | $y4^+$ | 383.222 | 506.261 | 385.226 | 506.261 | 14 |
| FLKPGVPDFK | | $y2^+$ | | 294.181 | | 294.181 | |
| | | $y4^{++}$ | | 253.634 | | 253.634 | |
| | | $b6^+$ | | 642.397 | | 648.411 | |
| CCoAOMT1.182-206 | $[M+3H^+]^{3+}$ | $y9^+$ | 867.104 | 953.487 | 869.109 | 959.501 | 31 |
| VGGLIGYDNTLWNGSVVAPPDAPMR | | $y7^+$ | | 783.382 | | 783.382 | |
| | | $y7^{++}$ | | 392.195 | | 392.195 | |
| CCoAOMT2.182-206 | $[M+3H^+]^{3+}$ | $y9^+$ | 858.432 | 927.472 | 860.437 | 933.485 | 31 |
| VGGLIGYDNTLWNGSVVAPADAPMR | | $y8^+$ | | 828.403 | | 828.403 | |
| | | $y7^+$ | | 757.366 | | 757.366 | |
| CCoAOMT3.104-115 | $[M+2H^+]^{2+}$ | $y9^+$ | 704.380 | 1044.609 | 707.888 | 1051.626 | 14 |
| EAYEIGLPFIQK | | $y8^+$ | | 915.566 | | 922.583 | |
| | | $y7^+$ | | 802.482 | | 809.499 | |
| | | $b4^+$ | | 493.193 | | 493.193 | |
| | | $b5^+$ | | 606.277 | | 606.277 | |
| CCoAOMT3.217-232 | $[M+2H^+]^{2+}$ | $y11^+$ | 873.956 | 1190.620 | 877.465 | 1197.637 | 27 |
| VEISQISIGDGVTLCR | | $y10^+$ | | 1077.536 | | 1084.553 | |
| | | $y9^+$ | | 990.504 | | 997.521 | |
| | | $y8^+$ | | 877.420 | | 884.437 | |
| | | $y4^+$ | | 549.281 | | 556.299 | |
| CCR2.299-308 | $[M+2H^+]^{2+}$ | $y8^+$ | 576.800 | 924.483 | 579.807 | 930.496 | 20 |
| DLGFEFTPVK | | $y6^+$ | | 720.393 | | 726.406 | |
| | | $y5^+$ | | 591.350 | | 597.364 | |
| | | $y3^+$ | | 343.234 | | 349.248 | |
| | | $b2^+$ | | 229.118 | | 229.118 | |
| | | $a2^+$ | | 201.123 | | 201.123 | |

TABLE SI - continued

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| COMT2.51-69 | $[M+3H^+]^{3+}$ | $y17^{++}$ | 628.667 | 878.467 | 631.006 | 881.976 | 20 |
| AGPGAFLSTSEIASHLPTK | | $y16^{++}$ | | 829.941 | | 833.450 | |
| | | $y14^{++}$ | | 765.912 | | 769.420 | |
| | | $y13^{++}$ | | 692.378 | | 695.886 | |
| | | $y18^{+++}$ | | 604.988 | | 607.327 | |
| | | $y17^{+++}$ | | 585.981 | | 588.320 | |
| HCT1.338-354 | $[M+2H^+]^{2+}$ | $y11^+$ | 944.015 | 1240.690 | 947.523 | 1247.707 | 28 |
| SALDFLELQPDLSALVR | | $y10^+$ | | 1111.647 | | 1118.664 | |
| | | $y9^+$ | | 998.563 | | 1005.580 | |
| | | $y8^+$ | | 870.504 | | 877.522 | |
| | | $b8^+$ | | 889.467 | | 889.467 | |
| | | $b9^+$ | | 1017.525 | | 1017.525 | |
| HCT1.338-354[c] | $[M+2H^+]^{2+}$ | $y11^+$ | - | - | 951.032 | 1254.724 | 28 |
| SALDFLELQPDLSALVR | | $y10^+$ | | - | | 1125.681 | |
| | | $y9^+$ | | - | | 1012.597 | |
| | | $y8^+$ | | - | | 884.539 | |
| | | $b8^+$ | | - | | 889.467 | |
| | | $b9^+$ | | - | | 1017.525 | |
| HCT6.338-354 | $[M+2H^+]^{2+}$ | $y11^+$ | 952.012 | 1240.690 | 955.521 | 1247.707 | 29 |
| SALDYLELQPDLSALVR | | $y10^+$ | | 1111.647 | | 1118.664 | |
| | | $y9^+$ | | 998.563 | | 1005.580 | |
| | | $y8^+$ | | 870.504 | | 877.522 | |
| | | $b8^+$ | | 905.461 | | 905.461 | |
| | | $b9^+$ | | 1033.520 | | 1033.520 | |
| PAL1.238-251 | $[M+2H^+]^{2+}$ | $y10^+$ | 740.378 | 1167.568 | 743.886 | 1167.568 | 24 |
| AAGIDSGFFELQPK | | $y9^+$ | | 1052.541 | | 1052.541 | |
| | | $y8^+$ | | 965.509 | | 965.509 | |
| | | $y6^+$ | | 761.419 | | 761.419 | |
| | | $y2^+$ | | 244.166 | | 244.166 | |
| | | a4+ | | 285.192 | | 292.209 | |
| PAL1.664-675 | $[M+2H^+]^{2+}$ | $y9^+$ | 623.830 | 875.483 | 627.338 | 882.500 | 15 |
| EELGTGLLTGEK | | $y7^+$ | | 717.414 | | 724.431 | |
| | | $y6^+$ | | 660.393 | | 667.410 | |
| | | $y5^+$ | | 547.309 | | 554.326 | |
| | | $y4^+$ | | 434.225 | | 434.225 | |
| | | $b2^+$ | | 259.092 | | 259.092 | |
| PAL2.661-672 | $[M+2H^+]^{2+}$ | $y9^+$ | 651.861 | 931.546 | 655.370 | 938.563 | 22 |
| EELGTILLTGEK | | $y7^+$ | | 773.477 | | 780.494 | |
| | | $y6^+$ | | 660.393 | | 667.410 | |
| | | $y5^+$ | | 547.309 | | 554.326 | |
| | | $y4^+$ | | 434.225 | | 434.225 | |
| | | $b2^+$ | | 259.092 | | 259.092 | |

TABLE SI - continued

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| PAL3.239-252 | $[M+2H^+]^{2+}$ | $y10^+$ | 747.385 | 1181.584 | 750.894 | 1181.584 | 24 |
| AAGIESGFFELQPK | | $y9^+$ | | 1052.541 | | 1052.541 | |
| | | $y8^+$ | | 965.509 | | 965.509 | |
| | | $y6^+$ | | 761.419 | | 761.419 | |
| | | $y2^+$ | | 244.166 | | 244.166 | |
| | | $a4^+$ | | 285.192 | | 292.209 | |
| PAL3.665-676 | $[M+2H^+]^{2+}$ | $y9^+$ | 644.853 | 917.530 | 648.362 | 924.547 | 14 |
| EELGTVLLTGEK | | $y7^+$ | | 759.461 | | 766.478 | |
| | | $y6^+$ | | 660.393 | | 667.410 | |
| | | $y5^+$ | | 547.309 | | 554.326 | |
| | | $y4^+$ | | 434.225 | | 434.225 | |
| | | $b2^+$ | | 259.092 | | 259.092 | |
| PAL4.614-622 | $[M+2H^+]^{2+}$ | $y8^+$ | 526.269 | 938.447 | 529.778 | 945.464 | 20 |
| IGSFEEELK | | $y7^+$ | | 881.425 | | 888.442 | |
| | | $y6^+$ | | 794.393 | | 801.410 | |
| | | $y5^+$ | | 647.325 | | 654.342 | |
| | | $y4^+$ | | 518.282 | | 525.299 | |
| PO1.136-149 | $[M+3H^+]^{3+}$ | $y8^+$ | 468.273 | 818.488 | 470.612 | 825.505 | 16 |
| DGIVSLGGPHIPLK | | $y7^+$ | | 761.467 | | 768.484 | |
| | | $y11^{++}$ | | 559.340 | | 562.849 | |
| | | $y10^{++}$ | | 509.806 | | 513.314 | |
| PO2.213-230 | $[M+2H^+]^{2+}$ | $y12^+$ | 1066.001 | 1442.651 | 1069.008 | 1442.651 | 31 |
| IYPTVDPTMDPDYAEYLK | | $y9^+$ | | 1113.510 | | 1113.510 | |
| | | $y8^+$ | | 998.483 | | 998.483 | |
| | | $y16^{++}$ | | 927.927 | | 930.934 | |
| | | $b6^+$ | | 689.350 | | 695.364 | |
| | | $[y16\text{-}b6]^+$ | | 413.203[d] | | 419.217[d] | |
| PO3.300-310 | $[M+2H^+]^{2+}$ | $y10^+$ | 582.788 | 1033.527 | 585.794 | 1039.541 | 19 |
| MSSITGGQEVR | | $y9^+$ | | 946.495 | | 952.509 | |
| | | $y8^+$ | | 859.463 | | 865.477 | |
| | | $y7^+$ | | 746.379 | | 752.393 | |
| | | $y6^+$ | | 645.331 | | 651.345 | |
| PO8.113-121 | $[M+2H^+]^{2+}$ | $y7^+$ | 553.298 | 887.483 | 556.807 | 894.500 | 20 |
| AFEIIEDLR | | $y6^+$ | | 758.441 | | 765.458 | |
| | | $y5^+$ | | 645.357 | | 652.374 | |
| | | $y4^+$ | | 532.273 | | 532.273 | |
| | | $b3^+$ | | 348.155 | | 348.155 | |

[a]Stable isotope-labeled amino acids are underlined. All labeled amino acids were uniformly labeled with $^{13}C$ and $^{15}N$ isotopes; [b]Collision Energy; [c]The double-labeled peptide (SIL**); [d]This is a secondary fragment ion to the y16 ion and contains the residues PTVD

## Supplemental Results

### Psuedo-First-Order Rate Reactions

The native, surrogate peptide during digestion can be viewed as the intermediate product in a reaction where it is first produced via proteolysis and, subsequently, decays into the final product (**Fig. S1A**).

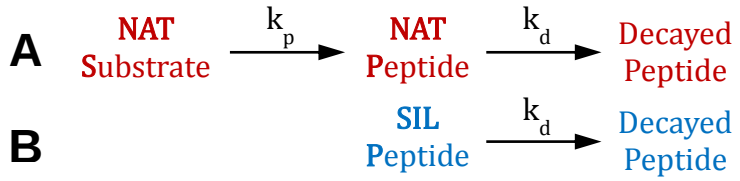


Fig. S1. **Elementary reaction schemes.** The terms $k_p$ and $k_d$ are the first-order rate constants for proteolysis and decay, respectively. The decay rate is assumed to be the same for both the (A) NAT and (B) SIL peptide.

If the concentration of the proteolytic enzyme remains constant, both the production and decay reactions can be assumed to follow pseudo-first-order kinetics (3), and the concentration of the intermediate, $P_{NAT}$, can be described using an integrated rate equation(4) that takes into account the simultaneous production and decay of the native peptide:

$$[P_{NAT}] = \frac{k_p}{k_d - k_p}\left(e^{-k_p t} - e^{-k_d t}\right)[S_{NAT}]_0 \qquad \textbf{Eq. S1}$$

In contrast, the SIL peptide standard can be viewed as the starting material in a simple decay reaction when it is not produced via proteolysis, as is the case in AQUA workflows(5). As such, the concentration of the SIL peptide can be described using an exponential decay function.

$$[P_{SIL}] = [P_{SIL}]_0 e^{-k_d(t+\Delta t)} \qquad \textbf{Eq. S2}$$

In this equation, the total time the SIL peptide undergoes decay is defined by sum of the digestion period, t, and the time difference, $\Delta t$, between the introduction of the SIL

peptide and the proteolytic enzyme. By this definition, Δt is positive if the SIL peptide is added before addition of the enzyme and negative if added after. Moreover, this equation assumes the SIL peptide decays at the same rate during the "pre-digestion" phase as it does during digestion, such that the same rate constant can be applied to the time periods t and Δt. It should be noted the use of pseudo-first-order kinetics here is an approximation because the dynamics of enzyme digestion are complicated by the presence of multiple substrates in a complex biological matrix.

In taking the quotient of **Eq. S1** and **Eq. S2**, respectively, the following equation is obtained to calculate the ratio of $[P_{NAT}]$ and $[P_{SIL}]$ as a function of time:

$$\frac{[P_{NAT}]}{[P_{SIL}]} = \frac{k_p}{k_d - k_p}\left(\frac{e^{-k_p t} - e^{-k_d t}}{e^{-k_d(t+\Delta t)}}\right)\frac{[S_{NAT}]_0}{[P_{SIL}]_0} \qquad \textbf{Eq. S3}$$

By this equation, the ratio measured by LC-MS ($[P_{NAT}]/[P_{SIL}]$) will only equal the true or expected ratio ($[S_{NAT}]_0/[P_{SIL}]_0$) under the following conditions: 1) the peptide must be produced much faster than it decays ($k_p >>> k_d$) and 2) the SIL peptide standard must be added concurrently with the proteolytic enzyme ($\Delta t = 0$). The simplified form of **Eq. S3** is shown in the body of the main manuscript (**Eq. 3**).

**Mathematical Regression Models**

Following quenching of the digestion, the ratio of the native and SIL peptide concentrations ($[P_{NAT}]/[P_{SIL}]$) is approximated using the corresponding ratio of their analytical signals ($A_{NAT}/A_{SIL}$). Thus, **Eq. 4** and **Eq. 5** from the main manuscript can be approximated by the following equations, respectively:

$$\left(\frac{A_{NAT}}{A_{SIL}}\right)[P_{SIL}]_0 = \frac{k_p}{k_d - k_p}\left(e^{-t(k_p - k_d)} - 1\right)[S_{NAT}]_0 \qquad \textbf{Eq. S4}$$

$$\left(\frac{A_{NAT}}{A_{SIL}}\right)[P_{SIL}]_0 = \frac{k_p}{k_d - k_p}\left(e^{-k_p t} - e^{-k_d t}\right)[S_{NAT}]_0 \qquad \textbf{Eq. S5}$$

By knowing the initial concentration of SIL peptide added to each sample, $[P_{SIL}]_0$, it is straightforward to calculate the value on the left side of these equalities. By plotting this value as a function of time, t, the constants ($k_p$, $k_d$, and $[S_{NAT}]_0$) can be determined through non-linear regression. **Eq. S4** was used for regression of the data obtained when the SIL peptides were added concurrently with the trypsin and **Eq. S5** was used for regression when SIL peptides were added post-digestion.

**Comparison of Measured and Regression Results**

According to the models, the concurrent SIL peptide introduction strategy (**Eq. S4**) will always result in a higher measurement ($A_{NAT}/A_{SIL}\times[P_{SIL}]_0$) than the post-digest strategy (**Eq. S5**). To validate the mathematical models accurately reflect the experimental data, the measured protein concentrations were compared to those determined by the regression analysis. More specifically, the differences in the protein concentrations measured between the two sets of digests were compared to the same differences calculated by their respective models. For the majority of detectable peptides, very good agreement was seen (**Fig. S2**); however, poor comparisons were made for a few peptides (CCoAOMT1.182-206, CCoAOMT2.182-206, CCoAOMT3.104-115, CCoAOMT3.217-232, and PAL1.238-251). Poor data was obtained for these peptides due to rapid degradation of the native peptide to near or below the detection limits at later time points, which resulted in poor regression analysis. Upon regression analysis, a Grubb's test was performed on the residuals of all data points, and those having significantly different residuals ($p$-value < 0.05) were excluded as outliers. After this, the regression analysis was repeated with the remaining data

points until no outliers were detected. Given the proteins/peptides that were determined to be outliers also had the lowest coefficients of determination during non-linear least squares regression ($R^2$, **Table I**), we believe this confirms the outlier testing was appropriate and unbiased.

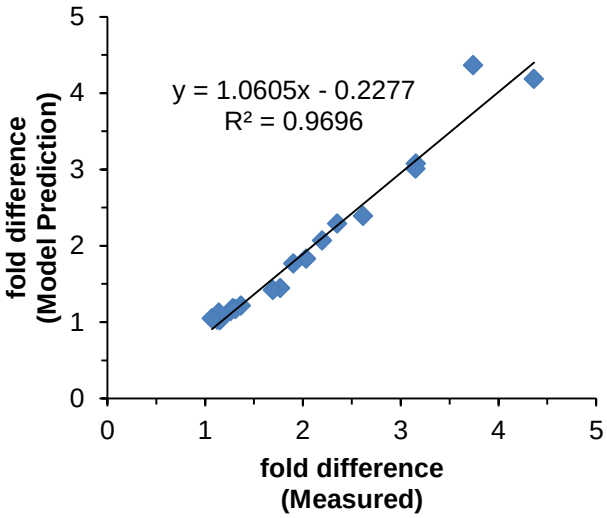


Fig. S2. **Plot comparing the fold differences of the concurrent and post-digest introduction strategies determined by measurement and regression.** The fold difference between the two strategies was calculated as the concurrent:post-digest ratio – notice, all measured ratios are greater than 1, indicating the concurrent introduction strategy always gave a larger value than the post-digest strategy. The slope of ~1 indicates the measured and predicted values correlate well, which suggests the mathematical models are able to explain the experimental results. The five peptides (listed above) were excluded from this figure as they were determined to be outliers.

**Relative Error Calculations**

Herein, relative error (RE) is defined as:

$$\mathrm{RE} = \frac{[S_{NAT}]_t - [S_{NAT}]_0}{[S_{NAT}]_0} \qquad \textbf{Eq. S6.1}$$

or,

$$\mathrm{RE} = \frac{[S_{NAT}]_t}{[S_{NAT}]_0} - 1 \qquad \textbf{Eq. S6.2}$$

In this case, $[S_{NAT}]_0$ is the true protein concentration prior to proteolysis and $[S_{NAT}]_t$ is the measured protein concentration after some time period of digestion. When the SIL peptides are added concurrently, the measured protein concentration is defined by **Eq. S4**, in which case **Eq. S6.2** becomes:

$$RE = \frac{\frac{k_p}{k_d - k_p}\left(e^{-t(k_p - k_d)}\right)[S_{NAT}]_0}{[S_{NAT}]_0} - 1 \qquad \textbf{Eq. S7.1}$$

which simplifies to give,

$$RE = \frac{k_p}{k_d - k_p}\left(e^{t(k_d - k_p)}\right) - 1 \qquad \textbf{Eq. S7.2}$$

This equation shows the relative error in the protein quantification is not dependent upon either the protein or peptide concentrations, but only on time and the rates of production and decay. Assuming the digest reaches completion ($t \rightarrow \infty$) and that peptide production is faster than peptide decay ($k_p > k_d$), the final relative error can be estimated by:

$$\lim_{t\to\infty} RE = \frac{k_p}{k_p - k_d} - 1 \qquad \textbf{Eq. S8}$$

Expressed as a percentage, this equation gives **Eq. 7**, which is shown in the main manuscript. It should be mentioned the relative error calculated by this equation ignores any experimental bias associated with the analytical measurements themselves.

**Theoretical Considerations for Proteolytic Production of SIL Peptides**

In PC-IDMS methods such as QconCAT(6) or PSAQ(7), the SIL peptide must also be released via proteolysis. In this manner, the SIL peptide behaves much like the native peptide and can be considered an intermediate reaction product (**Fig. S1A**). Under these circumstances the SIL peptide concentration follows an integrated rate equation analogous to that of the native peptide (**Eq. S1**) and the measured ratio of $[P_{NAT}]$ and $[P_{SIL}]$ can be described as the quotient:

$$\frac{[P_{NAT}]}{[P_{SIL}]} = \frac{\left(\frac{k_{p1}}{k_d - k_{p1}}\right)\left(e^{-k_{p1}t} - e^{-k_d t}\right)[S_{NAT}]_0}{\left(\frac{k_{p2}}{k_d - k_{p2}}\right)\left(e^{-k_{p2}} - e^{-k_d t}\right)[S_{SIL}]_0} \qquad \textbf{Eq. S9.1}$$

In this equation, $k_{p1}$ and $k_{p2}$ are the rate constants for the production of $P_{NAT}$ and $P_{SIL}$, respectively, and $[S_{SIL}]_0$ is the concentration of the substrate containing the SIL peptide. In the QconCAT method, the substrate would be the concatemer containing all the SIL peptide standards, while the substrate would be the SIL protein in the PSAQ method. Alternatively, the substrate could also be a cleavable SIL peptide(8). In all cases, the rate constant for decay, $k_d$, is still assumed to be the same for both the NAT and SIL peptides.

When the rates of production are near identical for both the NAT and SIL peptide ($k_{p1} \approx k_{p2}$), the ideal situation is observed in which the measured ratio, $[P_{NAT}]/[P_{SIL}]$, will equal the true ratio, $[S_{NAT})_0/[S_{SIL}]_0$ (**Fig. S3A**). This is thought to be the situation observed in the PSAQ method where the internal standard is a SIL protein. As the rates of production begin to deviate from each other, the measured ratios become biased relative to the true ratio (**Fig. S3B-C**).

It is important to note these results are contingent on the peptides undergoing decay. In the absence of decay ($k_d = 0$), the true ratio will always match the measured ratio so long as digestion conditions favor the production of the proper limit peptides(3) and the digestion is allowed to reach completion. When peptide decay does occur, the relative error can be approximated by:

$$\lim_{t\to\infty} \mathrm{RE} = \left(\frac{k_{p1}}{k_{p2}}\right)\left(\frac{k_{p2} - k_d}{k_{p1} - k_d}\right) - 1 \qquad \textbf{Eq. S10}$$

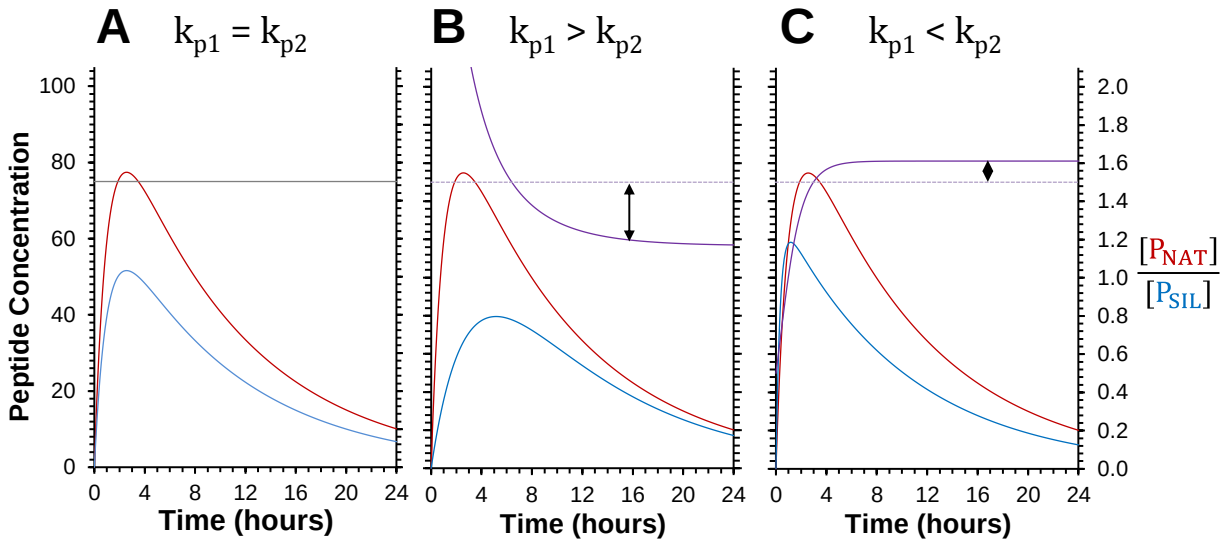


Fig. S3. **Theoretical models for peptide production and decay during PC-IDMS workflows that utilize proteolytic production of SIL peptides.** Shown are the modeled results when (A) $P_{NAT}$ and $P_{SIL}$ are produced at the same rate, (B) $P_{NAT}$ is produced faster than $P_{SIL}$, and (C) $P_{NAT}$ is produced slower than $P_{SIL}$. These plots were created using the following criteria. The blue and red lines in each plot indicates $[P_{NAT}]$ and $[P_{SIL}]$, respectively, while the dark purple line shows the measured ratio, $[P_{NAT}]/[P_{SIL}]$. The light purple dotted-line indicate the true ratio, $[S_{NAT}]_0/[S_{SIL}]_0$, which was set to 1.5 for each plot. Additional criteria used to create these plots were: $k_d$ = 0.1 $hr^{-1}$, $k_{p1}$ = 1.0 $hr^{-1}$, and the ratio of $k_{p1}$:$k_{p2}$ was: (A) 1:1 ($k_{p2}$=1 $hr^{-1}$), (B) 3:1 ($k_{p2}$=0.333 $hr^{-1}$) , and (C) 1:3 ($k_{p2}$=3 $hr^{-1}$).

This equation was derived using the same logic applied in the derivation of **Eq. S8** and shows the error is dependent upon the relative rates of NAT and SIL peptide production, as well as the relative rates of production and decay. Notice, the relative error will be negative if the NAT peptide is produced faster than the SIL peptide ($k_{p1} > k_{p2}$), which is consistent with the model's predicted underestimation (**Fig. S3B**). Under the opposite circumstances ($k_{p1} < k_{p2}$) the relative error will be positive due to an overestimation of the true ratio and, by that same token, overestimation of the true protein quantity (**Fig. S3C**). However, the error will be negligible when both peptides are produced much faster than they decay (or when $k_d \approx 0$).

**Peptide Production Times and Half-lives**

Utilizing the experimentally determined rate constants for peptide production and decay, the time required for complete production of each peptide can be calculated, as can each peptide's half-life (**Fig. S4** and **Table SII**). Under the digestion conditions employed all targeted peptides were fully produced within 9 hours and the majority (22 of 24) were fully produced in less than 4.5 hours. In contrast, the calculated half-lives for this same set of peptides spans a much broader dynamic range: 0.72 to 322 hours. Half of these (12 of 24) exhibit half-lives of less than 9 hours, which is the time required for complete production and, thus, "accurate" quantification of all proteins. In other words, half of the SIL peptides' concentrations will drop by 50% before the digestion period is completed. This finding explains why peptide stability, rather than the rate of proteolysis, is more likely to bias the results of a PC-IDMS assay.

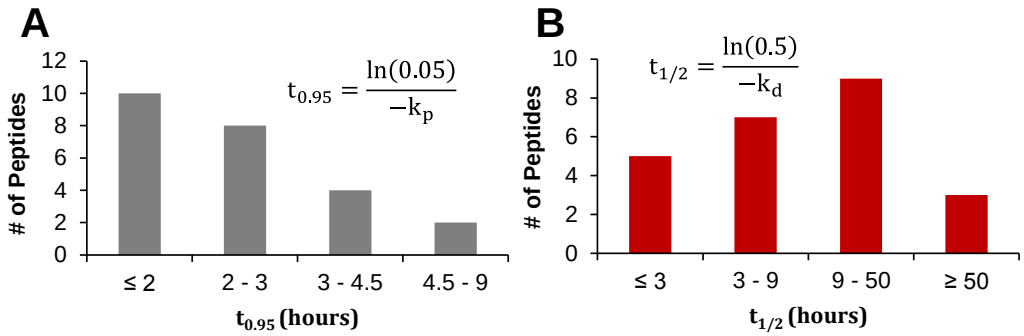


Fig. S4. **Distribution of peptide production times and peptide half-lives.** The peptide production times (A) were defined as the amount of time for 95% of the peptide to be produced via proteolysis and is denoted as $t_{0.95}$. The half-lives (B) were calculated according the typical definition (Ref. 3). The equations to calculate these values are shown.

TABLE SII
Peptide Half-lives and Production Times
Values were calculated based on experimentally determine rate constants

| Peptide | Sequence | $t_{1/2}$ [a] | $t_{0.95}$ [a] |
|---|---|---|---|
| 4CL3.262-273 | FDIGTLLGLIEK | 4.94 | 8.71 |
| 4CL5.262-273 | FEIGSLLGLIEK | 3.88 | 6.42 |
| C3H3.125-134 | VCTLELFSPK | 9.21 | 3.93 |
| C4H1.255-261 | DYFVDER | 206 | 2.09 |
| C4H2.255-261 | DYFVEER | 322 | 1.64 |
| CAD1.184-198 | GGILGLGGVGHMGVK | 3.77 | 2.96 |
| CAld5H1.426-435 | FLEPGVPDFK | 42.1 | 1.79 |
| CAld5H2.427-436 | FMKPGVPDFK | 15.2 | 1.52 |
| CAld5H2.L.427-436 | FLKPGVPDFK | 33.9 | 1.59 |
| CCoAOMT1.182-206 | VGGLIGYDNTLWNGSVVAPPDAPMR | 2.10 | 1.82 |
| CCoAOMT2.182-206 | VGGLIGYDNTLWNGSVVAPADAPMR | 1.02 | 1.84 |
| CCoAOMT3.104-115 | EAYEIGLPFIQK | 1.21 | 2.91 |
| CCoAOMT3.217-232 | VEISQISIGDGVTLCR | 0.72 | 1.20 |
| CCR2.299-308 | DLGFEFTPVK | 16.0 | 1.20 |
| COMT2.51-69 | AGPGAFLSTSEIASHLPTK | 9.77 | 3.40 |
| HCT1.338-354 | SALDFLELQPDLSALVR | 6.70 | 2.37 |
| HCT6.338-354 | SALDYLELQPDLSALVR | 7.66 | 2.26 |
| PAL1.238-251 | AAGIDSGFFELQPK | 1.25 | 3.05 |
| PAL1.664-675 | EELGTGLLTGEK | 49.6 | 1.25 |
| PAL2.661-672 | EELGTILLTGEK | 6.38 | 2.61 |
| PAL3.665-676 | EELGTVLLTGEK | 5.04 | 2.75 |
| PAL4\|5.614-622 | IGSFEEELK | 128 | 1.28 |
| PO1.136-149 | DGIVSLGGPHIPLK | 28.9 | 2.04 |
| PO8.113-121 | AFEIIEDLR | 36.0 | 4.27 |

[a]Units are hours

## Supplemental References


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