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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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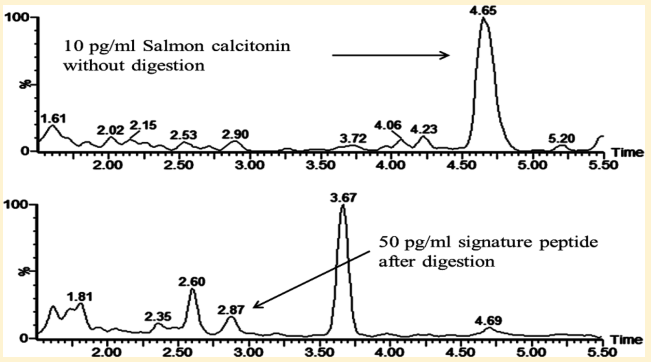
Supporting Information

ABSTRACT: Two important aspects of peptide and protein quantification by LC-MS/MS, the enzymatic digestion step and the internal standardization approach, were systematically investigated with a small protein, salmon calcitonin, which could be analyzed both without and with digestion. Quantification of undigested salmon calcitonin, after solid-phase extraction from plasma, resulted in a lower limit of quantification of 10 pg/mL, while introduction of a tryptic digestion step, followed by quantification of a signature peptide, increased this to 50 pg/mL. The sensitivity was reduced by interferences in the selected reaction monitoring (SRM) transition of the signature peptide due to the increase in sample complexity caused by the digestion and a less selective SRM transition of the signature peptide as compared to undigested salmon calcitonin. Eight internal standardization approaches were compared with respect to accuracy and precision in workflows with and without digestion. Analogue and stable-isotope-labeled (SIL) internal standards were evaluated including an in-house created ^{18}O-labeled peptide, a cleavable SIL peptide, and an internal standard created by differential derivatization of the signature peptide. We conclude that the best internal standard for the workflows both with and without digestion was the SIL form of the analyte, although the use of several SIL signature peptides and a differentially derivatized signature peptide also resulted in methods with performances which meet the FDA guidelines.



In recent years, liquid chromatography hyphenated to tandem mass spectrometric detection (LC-MS/MS) has gained popularity as an analytical platform for the targeted quantification of proteins, such as biomarkers and biopharmaceuticals, in complex biological samples, a field traditionally dominated by ligand binding assays (LBAs). This is driven by a number of analytical advantages over LBAs, including improved precision and accuracy, better robustness, and interlaboratory comparability of results and the potential for generic approaches without the need to raise antibodies against the target analyte.

Because of its incompatibility with molecules larger than approximately 10 kDa, protein quantification by LC-MS/MS usually includes proteolytic digestion of the analyte with an enzyme such as trypsin, to cleave it into a set of smaller peptides, one of which, the signature peptide, is subsequently used for quantification as a surrogate for the protein.

Much research has been devoted to the optimization of the enzymatic digestion step, particularly with regard to its speed, completeness and reproducibility and especially in the field of proteomics.[1−5] Still, the impact of digestion on the performance of targeted protein quantitation with regard to parameters such as selectivity and sensitivity has been relatively unexplored.

For both proteomic and targeted analytics, the proteolytic digestion of a biological sample such as plasma increases its complexity significantly, as each of the thousands of proteins is cleaved into multiple peptides with relatively similar physicochemical properties. Therefore, even when highly selective mass spectrometric detection is used in selected reaction monitoring (SRM) mode, other peptides may cause interfering peaks and elevate the background, both of which can reduce the obtainable sensitivity. In some cases, the presence of a signature peptide with favorable properties with regard to sensitivity and selectivity allows high-sensitivity quantification by digestion without further sample treatment,[4,6,7] but in other situations a more complex workflow may be required to reduce the number of interfering peptides and increase method sensitivity,[8−10] because sensitivity differences between various

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Table 1. Detected Species for All Workflows, Their Amino Acid Sequence, and, if Applicable, the Isotope Label

name	sequence
salmon calcitonin [1−32]	CSNLSTCVLGKLSQELHKLQTYPRTNTGSGTP-NH_2
human calcitonin [1−32]	CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP-NH_2
SIL-salmon calcitonin [1−32]	CSNLSTCVLGK*LSQELHKLQTYPRTNTGSGTP-NH_2 (* = $^{13}C_6$−$^{15}N_2$)
eel calcitonin [1−32]	CSNLSTCV LGKLSQELHKLQTYPRTDVGAGTP-NH2
salmon calcitonin [1−11]	CSNLSTCVLGK
human calcitonin [19−32]	FHTFPQTAIGVGAP-NH_2
SIL-salmon calcitonin [1−11][a]	CSNLSTCVLGK* (* = $^{13}C_6$−$^{15}N_2$)
^{18}O-salmon calcitonin [1−11]	CSNLSTCVLGK* (* = $^{18}O_2$)
derivatized salmon calcitonin [1−11]	*CSNLSTCVLGK* (* = $(CH_3)_2$)
diff. derivatized salmon calcitonin [1−11]	*CSNLSTCVLGK* (* = $(CHD_2)_2$)

[a]Added as is, or detected after proteolytic release from SIL-salmon calcitonine or the cleavable SIL peptide.

peptides from the same target protein may be as large as a factor 1000.[11]

One of the most advantageous features of the LC-MS/MS platform is the possibility of quantifying multiple compounds simultaneously. This enables the use of internal standards, which enhance the accuracy and precision of the analytical method by offering correction for analytical variability due to the sample handling steps. Compared to the relatively straightforward situation for small molecules, for which either stable-isotope-labeled (SIL) forms of the analyte or close structural analogues are used, internal standardization for proteins is more complex. A multitude of different internal standardization approaches have been described in the field of protein quantification with LC-MS/MS, and they all have their theoretical advantages and disadvantages.[12] The most ideal situation would be the use of a SIL form of the intact protein[13] or of a structurally closely related protein[14] to correct for as much of the analytical workflow as possible. Another, more widely used approach is the use of a SIL form of the signature peptide,[15,16] which will only cover the postdigestion part of the analysis. In order to correct for the digestion, at least partially, the use of a SIL peptide containing one or more cleavable groups has been described.[17,18] An interesting possibility is the preparation of an ^{18}O-labeled form of the signature peptide by isotope exchange with ^{18}O-labeled water, which can be performed in any laboratory without specialized equipment,[19] as long as oxygen back-exchange during sample processing or extract storage is avoided.[20] Finally, preparation of a SIL internal standard by chemical derivatization with a SIL reagent during sample preparation by so-called differential labeling is another option.[6]

Although the different internal standardization approaches for use in quantitative proteomics and target analysis[12,18,21] have been reviewed in the literature and a few research papers have included a comparison of two[22,23] or three[24] internal standardization approaches for their specific application, a systematic experimental investigation into the relative merits of all different types of internal standards has not been performed. In this paper, we report the results of such an investigation using the small biopharmaceutical protein salmon calcitonin as model compound. Since salmon calcitonin can be quantified by LC-MS/MS both with and without digestion, the impact of eight different internal standardization methods as well as the effect of the enzymatic digestion step on method performance (sensitivity, selectivity, precision, and accuracy) was investigated.

EXPERIMENTAL SECTION

Chemicals. Ultrapure water was produced using an in-house purification system (Sartorius, Göttingen, Germany). Acetonitrile and methanol were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonia, Tween-20, ammonium bicarbonate, trypsin (T0303), pyridine-borane complex, formaldehyde, hydrochloric acid (37%), soybean trypsin inhibitor, and ^{18}O-labeled water, all of analytical purity, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Heptafluorobutyric acid (HFBA) and formic acid were purchased from Merck (Whitehouse Station, NJ, USA). Salmon, eel, and human calcitonin were obtained from Bachem (Bubendorf, Switzerland). SIL-salmon calcitonin, SIL-salmon calcitonin signature peptide [1−11], and the cleavable SIL-salmon calcitonin peptide [1−11] were purchased from JPT-peptides (Berlin, Germany). Human plasma was purchased from Seralabs (West Sussex, U.K.). The amino acid sequences of the peptides are presented in Table 1.

Preparation of Stock Solutions, Calibration Standards and Validation Samples. A stock solution of 1 mg/mL salmon calcitonin was prepared in a solution which contained 10% acetonitrile, 10 mM ammonium formate, and 0.001% Tween-20 in water. Standard solutions at 20.0 μg/mL and 500, 50.0, and 5.00 ng/mL were prepared in the same solvent.

Calibration and quality control samples were prepared using a single source of human plasma whereas matrix variability samples were prepared using six independent sources of human plasma; in all cases, the anticoagulant was K_2EDTA. Three different calibration ranges were used: 10−1000 pg/mL (workflow **A**), 50−5000 pg/mL (workflow **B**), and 100−10000 pg/mL (workflow **C**). The preparation details of calibrators, quality control samples, and samples to test matrix variability are summarized in Table S1 of the Supporting Information. After preparation, all samples were stored at −20 °C.

^{18}O-Labeling of the Signature Peptide. First, 200 μL of salmon calcitonin stock solution was digested for 2 h at 37 °C after addition of 100 μL of 100 mM ammonium bicarbonate (pH 8.2) and 50 μL of a 1-mg/mL trypsin solution in 1 mM hydrochloric acid, to release the signature peptide. The digestion was stopped by the addition of 25 μL of HFBA. To remove trypsin and the digestion buffer from the digested sample, reversed-phase solid-phase extraction (SPE) was applied as follows. A Waters (Milford, MA, USA) Oasis HLB 30-mg, 1-mL cartridge was conditioned with 1 mL of methanol followed by 1 mL of 0.4% HFBA in water. The digested salmon calcitonin sample was diluted to 1 mL with 0.4% HFBA in water and loaded on the cartridge, which was subsequently

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washed with 1 mL of 30% methanol and 0.2% HFBA in water. Elution was performed with 1 mL of 90% methanol and 0.2% HFBA in water. The eluate was collected in a silanized glass tube and evaporated to dryness at 60 °C under a gentle stream of nitrogen. The dried sample was reconstituted in 250 mg of ^{18}O-labeled water, transferred to a 1.5 mL tube which, after addition of 2 μL of HFBA, was capped and placed at 50 °C for 48 h. The process of oxygen exchange was stopped by raising the pH to 6−7 by addition of 200 μL of 250 mM ammonium bicarbonate. The resulting sample was diluted to a volume of 1 mL with water and stored at −20 °C.

Workflows. Three different analytical workflows for peptide and protein quantification were investigated (Figure 1).

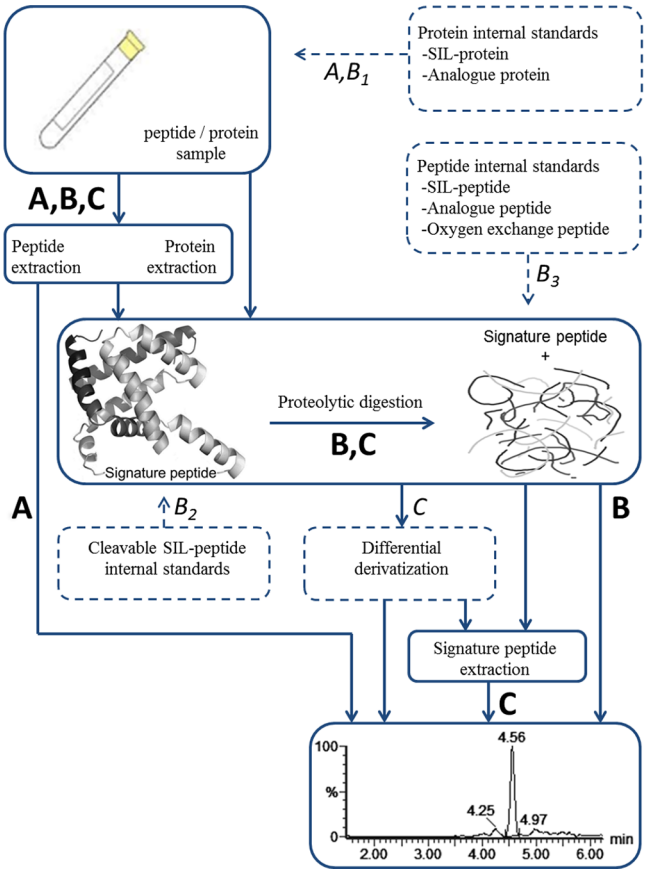

Figure 1. Graphical representation of workflows **A**−**C** and the different internal standardization approaches. The different steps of each of the three workflows, **A** (extraction), **B** (extraction and digestion), and **C** (extraction, digestion, and derivatization), are indicated in bold. The different points at which the internal standards were added to these respective workflows are shown in italic letters (*A*, B_{1-3}, and *C*).

Workflow **A** comprises only an extraction (SPE) from plasma followed by analysis of the undigested analyte, whereas **B** incorporates a subsequent trypsin digestion step and analysis of a signature peptide and **C** has an additional derivatization step, cleanup by ion-exchange SPE and LC-MS/MS analysis of the derivatized signature peptide. In workflow **A**, the following internal standards were added to the plasma sample prior to SPE: *A*, intact stable-isotope-labeled calcitonin; eel calcitonin (a close analogue) and human calcitonin (a more distant analogue). In workflow **B**, internal standards were added at three separate points in the flow-scheme. At B_1, intact human calcitonin and SIL-salmon calcitonin were added to the sample before the extraction; at B_2, a cleavable SIL signature peptide was added to the sample directly before digestion, while the SIL signature peptide, the analogue peptide, and the ^{18}O-labeled signature peptide were added after completion of the digestion at B_3. In workflow **C**, the internal standard was created by derivatization of a digest of a standard solution of salmon calcitonin with d_2-formaldehyde in parallel to the derivatization of the digests of plasma samples with unlabeled formaldehyde.

Accuracy and precision data were obtained by performing a one-run validation for each internal standard, by analysis of a calibration curve, matrix variability samples at the lower limit of quantification (LLOQ) in six different lots of plasma, and validation samples at four concentration levels spread over the calibration range, each in 6-fold. For efficiency reasons, the internal standards were combined in one validation run, where possible. Accuracy was expressed as the bias of a mean result from its corresponding nominal concentration and precision as the coefficient of variation (CV) obtained for the six replicates. Acceptance criteria were as per international guidelines for regulated bioanalysis: bias and coefficient of variation had to be within 15% (20% at the LLOQ).

Analyte Extraction from Plasma. Solid-phase extraction, using Oasis HLB 30 mg 1 mL cartridges (Waters), was applied to extract the analyte. A cartridge was conditioned with 1 mL of methanol and 1 mL of 0.4% HFBA in water. Next, 500 μL of plasma, mixed with 500 μL of aqueous 0.4% HFBA was loaded onto the cartridge. The cartridges were subsequently washed with 1 mL of 0.4% HFBA in water followed by 1 mL of a mixture of 30% methanol and 0.2% HFBA in water. Elution was performed with 1 mL of 90% methanol and 0.2% HFBA in water. The eluate was evaporated to dryness at 60 °C under nitrogen and reconstituted in 150 μL of a mixture of 10% of acetonitrile and 0.001% of Tween-20 in water. These extracts were transferred either to an injection-vial for direct analysis, in workflow **A**, or to a 1.5-mL polypropylene tube for tryptic digestion in workflows **B** and **C**.

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; see Supporting Information.

Tryptic Digestion. To 150 μL of plasma extract, 50 μL of 250 mM ammonium bicarbonate buffer (pH ∼8.2) and 50 μL of 1 mg/mL trypsin in 1 mM hydrochloric acid were added. The samples were placed at 37 °C and shaken at 750 rpm for 2 h. The digestion was stopped by the addition of 50 μL of 1% formic acid and 10% methanol in water, for direct analysis in workflow **B** or, in case of the ^{18}O-labeled internal standard, by the addition of 50 μL of a 10 mg/mL solution of soybean trypsin inhibitor in water. To the samples which were to be derivatized in workflow **C**, the digestion was stopped by the addition of the derivatization reagents.

Differential Derivatization. Due to the toxic, volatile, and pungent chemicals required for the derivatization reaction, the following procedures were carried out in a fume hood. To the digest of a standard solution of salmon calcitonin and to the digests of extracted plasma samples was added, respectively, 400 μL of a solution containing 10% of deuterated or unlabeled formaldehyde and 10% pyridine−borane complex in methanol. The samples were incubated at 37 °C while being shaken at 750 rpm for 1 h. After derivatization with deuterated formaldehyde, the internal standard sample was diluted to 5.0 mL with methanol in water 50% (v/v), of which 50 μL was added to the

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derivatized plasma digests of the analytical batch. These samples were subsequently transferred to a silanized glass tube and evaporated to dryness at 60 °C under a gentle flow of nitrogen in approximately 1 h.

Clean-up of Derivatized Calcitonine Signature Peptide from the Derivatization Residue. Waters Oasis MCX 30 mg, 1 mL cartridges were conditioned with 1 mL of methanol and 1 mL of 1% formic acid in water. Next, the derivatized sample, reconstituted in 1 mL of 30% methanol and 1% formic acid in water, was loaded onto the cartridge, which was subsequently washed with 1 mL of 1% formic acid in water and 1 mL of acetonitrile and eluted with 1 mL of 60% methanol and 2% ammonia in water. The eluate was collected in a glass tube and evaporated to dryness at 60 °C under nitrogen. The dried extract was reconstituted in 150 μL of 10% of acetonitrile and 0.001% of Tween-20 in water.

Chromatographic Separation and Detection. The chromatographic system consisted of an Acquity I-class UPLC system (Waters) and a 100 mm × 2.1 mm i.d., 1.7-μm particle diameter Acquity CSH-C18 analytical column (Waters) thermostatted at 45 °C. The mobile phase, a mixture of 0.1% formic acid in water (A) and acetonitrile (B) was delivered at a flow rate of 0.500 mL/min. Detection was performed with a TQ-S triple quadrupole mass spectrometer (Waters).

The extracts resulting from each of the three different workflows required a separate set of optimal chromatographic and mass spectrometric conditions. The SRM transitions and MS/MS settings used for detection for each of the (signature) peptides are summarized in Table S2 of the Supporting Information. Injection volumes were 20, 35, and 20 μL for workflows **A**, **B**, and **C**, respectively. A mobile phase gradient of 17.5% B to 27.5% B in 10 min was used for workflow **A**, while isocratic elution was performed at 12% B for workflows **B** (8 min) and **C** (5.5 min). In all cases, a step gradient at 90% was applied afterward for 1 min, followed by equilibration at initial conditions for 2 min.

RESULTS AND DISCUSSION

Selection of the Test Substance. Selection of the 32-amino acid peptide salmon calcitonin, with an average molecular mass of 3431.9 Da, was based on several considerations. First, it is amenable to proteolytic digestion with trypsin as its sequence contains three trypsin cleavage sites. This qualifies the molecule as a test substance for the comparison of workflows and internal standardization approaches which are typical for protein quantification and include a tryptic digestion step. Second, the molecule is small enough to allow direct quantification of the intact molecule by LC-MS/MS, which enables the addition of a typical peptide quantification workflow (i.e., without a digestion step). In this way, the effect of the digestion step on method performance can be assessed.

Finally, the availability of internal standard candidates is relatively good because analogue molecules (calcitonins from other species such as eel and human) are commercially available and due to its relatively small size, a stable-isotope-labeled (SIL) internal standard of the entire molecule can be readily obtained by chemical synthesis.

Extraction of the Analyte from Plasma. As discussed in the next section, enrichment of the analyte and removal of interfering matrix compounds is important for high-sensitivity analysis. Reversed-phase SPE was applied to extract intact salmon calcitonin as well as the different intact internal standards: SIL-salmon calcitonin, eel, and human calcitonin from human plasma. During the extraction, an anionic ion-pairing reagent (HFBA) was added, which pairs with positively charged amines on the analyte and, due to its acidic nature, also protonates the carboxylic acid moieties present on the analyte. The charge-neutral complex thus formed showed increased retention compared to the otherwise zwitterionic peptide. HFBA is relatively volatile, which allowed its removal by the evaporation step that followed the SPE procedure. This is important, because the acidity of HFBA is incompatible with the subsequent digestion and its presence may also reduce ionization efficiency upon LC-MS/MS analysis.

After trapping the peptides on the cartridge, washing with 30% methanol removed interfering substances without eluting any significant amount of the analyte or the internal standards. Furthermore, when using 90% methanol, elution was essentially complete. The extraction recovery of the analyte and the three corresponding intact internal standards was approximately 80%. A total protein assay showed that more than 99% of plasma proteins were removed by this SPE procedure.

Digestion—Impact on Method Selectivity and Sensitivity. Even though several proteolytic enzymes are available, trypsin appears to be most widely used to cleave analytes and internal standards into peptides,[12] probably because of the favorable average length of the peptides it releases and their good ionization characteristics for electrospray LC-MS/MS, compared to peptides generated by other proteolytic enzymes.[25]

Tween-20, a polysorbate surfactant, was added to the digestion solvent to prevent adsorption of salmon calcitonin and its signature peptide to the surface of the reaction tube and the autosampler vial.[26] The digestion was found to be complete after 1 h, as is shown in Figure S1 in the Supporting Information. However, to account for a possible variability between samples, a digestion time of 2 h was used.

The tryptic digestion step may impact the selectivity of an LC-MS/MS method for a protein analyte in a protein-containing matrix, because proteins present in the sample will be cleaved into a mixture of peptides, which are much more similar to each other in terms of size and physicochemical properties than the proteins in the original sample.

The effect of the digestion step on the selectivity and sensitivity of this type of quantifications was assessed by mapping the effect of adding increasing amounts of digested plasma to a digested aqueous solution of salmon calcitonin (Figure 2). LC-MS/MS chromatograms are shown of a tryptic digest of an aqueous solution of salmon calcitonin at 2.00 ng/mL to which increasing amounts of a tryptic digest of untreated plasma (1−50%) were added. In the aqueous sample, only one peak is visible, which corresponds to the signature peptide [1−11] of calcitonin (A). After addition of 1% plasma digest, the presence of other peptides originating from digested matrix proteins increased the background visibly (B). At higher percentages of digested plasma, the background increased further and more interfering peaks appeared in the chromatograms (C, D, and E). Finally, at 50% of added digested plasma, the analyte peak can no longer be distinguished from plasma interferences (F). Despite the addition of increasing amounts of digested plasma, the height of the salmon calcitonin peak did not change. This indicates that the presence of a high concentration of peptides, part of which coeluted with the peptide of interest, did not cause ion-suppression. Instead,

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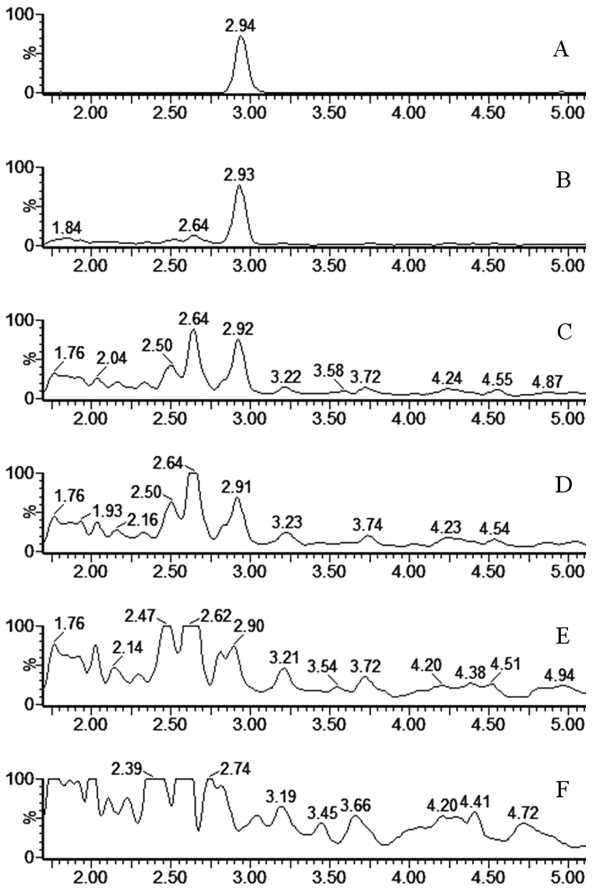

Figure 2. LC-MS/MS chromatograms in the SRM mode for tryptic peptide [1−11] (retention time approximately 2.9 min). Increasing amounts (volume percentage) of trypsin-digested plasma were added to a digest of an aqueous solution of salmon calcitonin at 2.0 ng/mL: 0% (A), 1% (B), 5% (C), 10% (D), 20% (E), and 50% (F); all chromatograms were scaled to the height of the peptide peak.

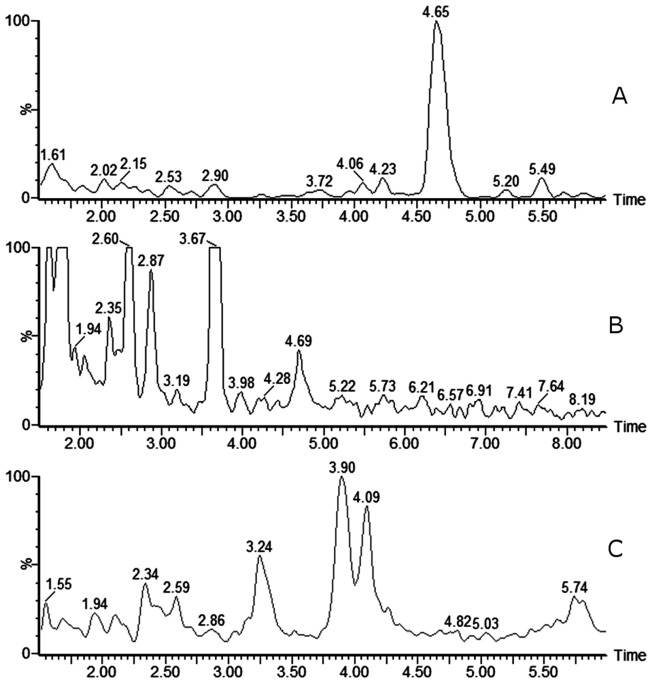

Figure 3. LC-MS/MS chromatograms of human plasma samples spiked with salmon calcitonin at the LLOQ level for each of the three workflows: **A**, intact salmon calcitonin (4.65 min) after extraction from plasma (10 pg/mL); **B**, tryptic peptide [1−11] (2.87 min.) after extraction of salmon calcitonin (50 pg/mL) and digestion; **C**, derivatized tryptic peptide [1−11] (3.90 min) after extraction from plasma (100 pg/mL), digestion, and derivatization (C).

sensitivity was limited by an increase in the background signal, which reduced the signal-to-noise ratio and thus the sensitivity of the method.

This shows that the high complexity of a plasma digest may result in selectivity limitations due to the presence of other tryptic peptides, even for a highly selective detection technique such as LC-MS/MS in the SRM mode. In terms of sensitivity, we estimated that the lower limit of quantification, defined for this purpose as the analyte response corresponding to 10 times the background, increased from 0.2 ng/mL for a clean aqueous solution to 20 ng/mL in the presence of 50% plasma digest, i.e. a loss of a factor of 100 in method sensitivity.

Chromatography and Detection—Impact on Method Selectivity and Sensitivity. Because of the interference of codigested matrix proteins, it is important to optimize the LC-MS/MS conditions in such a way that their impact is minimized. For all workflows, the chromatography was fine-tuned to obtain a maximum separation between the (non-endogenous) analyte peak and interfering peaks originating from endogenous matrix proteins within a reasonable run time (8.5−13 min). This was achieved by comparing the response for blank plasma at the retention time of the analyte to that of the analyte itself. The overall effect of digestion on selectivity and sensitivity is illustrated in Figure 3, which shows the LC-MS/MS chromatograms at the LLOQ for samples from workflows **A** and **B** (see Figure 1). In Figure 3A, the LC-MS/MS chromatogram of a plasma extract from workflow **A** (intact salmon calcitonin) is shown, which allows an LLOQ of 10 pg/mL. Figure 3B shows the chromatogram of an identical plasma extract, analyzed after trypsin digestion (workflow **B**), which resulted in a considerably higher LLOQ of 50 pg/mL. The sensitivity of workflow **B** is limited by the presence of significant interferences, which are not present for workflow **A**. This is explained by the increased complexity of the sample after digestion, combined with a less selective SRM transition. Even though 99% of the protein content was removed from the samples before the digestion step, the remaining 1% (~650 μg/mL) of plasma proteins is still more than a factor of 10^6 higher than the LLOQ.

Differential Derivatization—Impact on Method Selectivity and Sensitivity. Derivatization of a tryptic digest with formaldehyde can improve the physicochemical properties of the signature peptide for LC-MS/MS analysis, as it may result in increased chromatographic retention and enhanced ionization and fragmentation, resulting in an increase in sensitivity of the analytical method.[27]

For digested salmon calcitonin, however, derivatization of the signature peptide with formaldehyde (workflow **C** in Figure 1) did not increase the sensitivity of the method. On the contrary, the LLOQ increased to 100 pg/mL, the least favorable for the three workflows and 2-fold higher than for workflow **B**, which uses digestion without derivatization. Even though an additional SPE step was included to further purify the extract after derivatization and a high-resolution chromatographic method was applied, the sensitivity-limiting factor in this case was the presence of significant interferences resulting in a high background (see Figure 3C). A comparison of the selectivity

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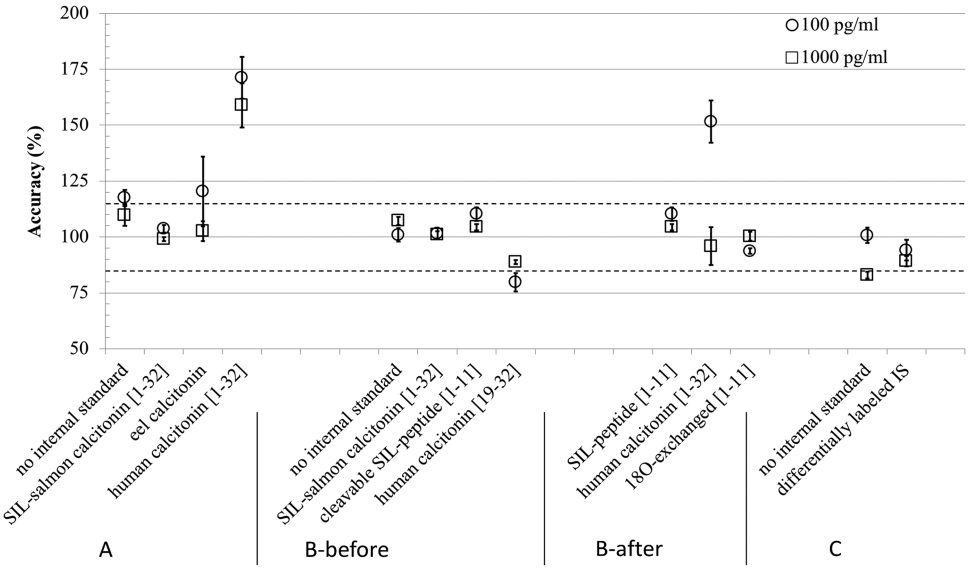

Figure 4. Precision (expressed as error bars) and accuracy for ten different internal standardization approaches according to workflows **A**–**C** (see Figure 1) as compared to the same workflow without internal standard. The results were obtained by 6-fold analysis of the 100 and 1000 pg/mL samples. The dotted lines indicate the acceptable 15% accuracy limits as set by the FDA-guidance. In workflow **B**, internal standards were either added before or after the digestion step.

of the available SRM transitions for the derivatized signature peptide can be found in Figure S2 of the Supporting Information.

Preparation of an ^{18}O-Labeled Internal Standard. In-house preparation of an ^{18}O-labeled form of a signature peptide is an interesting option to obtaining a SIL internal standard, as an alternative to purchasing it from commercial suppliers. In the most widely used approach, acid-catalyzed oxygen exchange,[19] the peptide is heated in acidified $H_2{}^{18}O$ to exchange all carboxyl ^{16}O atoms from the C-terminus and the aspartic and glutamic acid moieties in the peptide with the ^{18}O atoms of the water molecules. After heating a digest of salmon calcitonin in acidified $H_2{}^{18}O$ at 50 °C for 48 h, the exchange was found to be complete, since the unlabeled form of the signature peptide could no longer be detected by LC-MS/MS. An undesirable side-reaction is the possible occurrence of deamidation of glutamine and asparagine under the reaction conditions, which lowers the yield of the reaction product and gives rise to deamidated side-products which may need to be chromatographically separated.[28] Our signature peptide contains one asparagine residue, and after completion of the oxygen-exchange reaction, the deamidated product of the labeled signature peptide was found to be present but not to interfere because it had been chromatographically separated.

When using internal standards created with this approach, back-exchange of the ^{18}O atoms with ^{16}O atoms of the water molecules in the sample may occur. This will happen when the ^{18}O-labeled internal standard is exposed to low pH values or to active trypsin[27] during sample processing or storage of the extracts in the autosampler. Several approaches to prevent back-exchange were compared (see Figure S3, Supporting Information). In our work, back-exchange was prevented by the addition of soybean trypsin inhibitor at twice the molarity of trypsin, which inhibited trypsin without acidification.

Internal Standard Selection—Impact on Method Precision and Accuracy. Tables S3–S6 (Supporting Information) summarize all results obtained for the different internal standardization approaches, including those for the three workflows without an internal standard. The values for accuracy and precision for the QC samples with the different workflows and internal standards are shown (Figure 4).

Results for workflow **A** (quantification of the intact analyte) without an internal standard indicate that sample handling and extraction introduced too much variability at 100 pg/mL, resulting in a bias outside the acceptance criterion of ±15%. When SIL-salmon calcitonin was used as internal standard, a large improvement in both accuracy and precision was observed at both high and low concentrations. This finding is in line with the expectation that a SIL internal standard of the analyte itself will offer optimal correction for experimental variability. Applying eel calcitonin, a close analogue of salmon calcitonin (90% sequence identity), as internal standard, did not improve overall assay performance. While precision and accuracy were acceptable at the higher analyte concentration, the internal standard introduced additional variability at the lower concentration, resulting in reduced precision. Using human calcitonin (50% sequence identity) as internal standard reduced method performance: both precision and accuracy were inferior to the situation without any internal standard, which illustrates that this compound did not correct for variability but rather introduced it into the assay. Here it should be noted that, by the analysis of blank human plasma samples in each analytical run, it was shown that endogenous levels of human calcitonin in plasma were too low to be detected and thus did not interfere.

In workflow **B** (quantification of the digested analyte), a distinction was made between internal standards added before and after digestion. These correspond to internal standards that are codigested (B-before) and are expected to cover the digestion step, and to internal standards that are SIL or structural analogue forms of the signature peptide and will only cover the postdigestion part of the analysis (B-after). Because of its structural similarity, tryptic digestion of eel calcitonin results in the formation of a peptide which is identical to the signature peptide of salmon calcitonin, whence this compound is not suitable as an internal standard for a workflow including

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digestion. Just as for the quantification of the intact analyte, human calcitonin was a relatively poor performer both after its codigestion to its signature peptide [19−32] (B-before) and when added postdigestion [1−32] (B-after). All other approaches tested in workflow **B**, including the omission of an internal standard, generated acceptable results, which shows that in this workflow all steps were well under control. Again, the best results in terms of precision and accuracy were obtained when a SIL form of either the intact analyte, SIL-salmon calcitonin [1−32], or the signature peptide, SIL peptide [1−11], were added. As has been reported before,[18] the use of a cleavable SIL peptide internal standard did not improve method performance compared to the SIL peptide [1−11] internal standard, which illustrates that, in this case, the digestion step did not negatively impact method performance. This conclusion is supported by the observation that there was no difference between the performance of a SIL form of the intact analyte and that of a SIL signature peptide for workflow **B**.

For protein quantification by LC-MS/MS, which includes a digestion step, this indicates that the use of a SIL form of the intact protein, which is more difficult to obtain, does not have clear advantages over the use of the SIL form of the signature peptide, as long as the digestion step is well optimized and controlled. Another important observation is that a commercially obtained SIL peptide internal standard and the in-house prepared ^{18}O-labeled form performed comparably. Therefore, when due precautions are taken to avoid isotope back-exchange, which in our work was achieved by adding soybean trypsin inhibitor to stop the digestion, the preparation of an ^{18}O-labeled peptide can be a straightforward and economical alternative to a chemically synthesized SIL peptide.

In workflow **C** (quantification of digested and derivatized analyte), it was seen that using a differentially labeled internal standard slightly improved assay performance compared to the results without internal standard. Since the derivatized internal standard is added only after most sample handling steps (extraction, digestion, derivatization) have been performed, it corrects only for variability of the cleanup step and the LC-MS/MS analysis. Precision and accuracy of this internal standardization approach are comparable to the SIL peptide and ^{18}O-labeled peptide approaches for workflow **B**, which do not include a derivatization and second SPE step. This implies that these approaches are equivalent in terms of method performance, albeit that workflow **C** is more laborious and therefore will have a higher risk of experimental variability.

CONCLUSIONS

From the presented results, several conclusions can be drawn that apply to both peptide and protein quantifications with LC-MS/MS, although it should be realized that the situation may vary from analyte to analyte. The technique is suitable for high-sensitivity analysis of complex biological samples, as demonstrated by the low pg/mL LLOQ for both intact and digested salmon calcitonin in human plasma. In the case of quantification of peptides, selectivity and, as a result, sensitivity may be reduced when an enzymatic digestion step is introduced as part of the analytical approach. This is caused by the release of a multitude of peptides from matrix proteins, that have physicochemical properties similar to the analyte and interfere in the chromatograms. To obtain high sensitivity for proteins, which typically requires a digestion step, reducing the complexity of the digest by a rigorous cleanup of the biological matrix is important to remove interfering peptides from the final extract. Our experiments showed that the sensitivity of an analytical method was reduced by a factor of 100 by the presence of residual matrix proteins during digestion. Still, even when most of the matrix background is eliminated from the sample, a loss in sensitivity may be encountered. In the case of salmon calcitonin in plasma, over 99% of the plasma proteins were removed, but upon digestion sensitivity, they still decreased by a factor 5 compared to the digestion-free approach.

Method performance in terms of accuracy and precision depends on the choice of the internal standard. When all steps of an analytical procedure, including digestion, extraction, or derivatization, are well-controlled and optimized, the need for an internal standard that covers all steps may not be absolute. In the case of salmon calcitonin quantified after digestion, the most favorable results were obtained using a SIL form of the intact analyte, but the performance of a SIL form of the signature peptide was equally acceptable. For protein quantification, this indicates that protein-based internal standards are not necessarily superior to the more easily accessible peptide-based ones. Although the use of an ^{18}O-exchanged peptide internal standard imposes certain limitations on an analytical method, because of the need to prevent oxygen back-exchange, the straightforward and relatively quick procedure to create such a standard and its demonstrated good corrective abilities make this an attractive and cost-effective approach. In our hands, the use of differential derivatization to create a SIL internal standard was the least optimal, in terms of both obtained sensitivity and complexity of the workflow. Therefore, although it is a generic and relatively cheap way to obtain an internal standard, this approach does not seem to have much added value compared to, for example, the creation of an ^{18}O-labeled peptide.

ASSOCIATED CONTENT

Supporting Information

Description of the total protein assay; figures showing digestion time course, SRM transition selectivity, and back-exchange prevention approaches; and tables of preparation of plasma samples, the MS settings used, and accuracy and precision results for workflows A, B-before, B-after, and C. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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