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Published in:
Bioanalysis

DOI:
10.4155/bio-2023-0129

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2023

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Sleumer, B., Kema, I. P., & van de Merbel, N. C. (2023). Quantitative bioanalysis of proteins by digestion and LC-MS/MS: the use of multiple signature peptides. *Bioanalysis*, *15*(19), 1203-1216. https://doi.org/10.4155/bio-2023-0129

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Review

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Bioanalysis

Quantitative bioanalysis of proteins by digestion and LC–MS/MS: the use of multiple signature peptides

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The use of multiple signature peptides for the quantification of proteins by digestion and LC–MS/MS is reviewed and evaluated here. A distinction is made based on the purpose of the use of multiple peptides: confirmation of the protein concentration, discrimination between different protein forms or species and *in vivo* biotransformation. Most reports that describe methods with at least two peptides use these for confirmation, but it is not always mentioned how the peptides are used and how possible differences in concentration between the peptides are handled. Differences in concentration are often reported in the case of monitoring different protein forms or *in vivo* biotransformation, and this offers insight into the biological fate of the protein.

First draft submitted: 30 June 2023; Accepted for publication: 10 August 2023; Published online: 19 September 2023

Keywords: bioanalysis • confirmation • digestion • LC–MS/MS • protein biotransformation • protein isoforms • protein quantification • signature peptides

Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is increasingly used for the quantification of proteins in biological samples as an alternative for or in addition to ligand-binding assays (LBAs). Due to its different analytical principles, LC–MS/MS can provide complementary qualitative and quantitative information with high accuracy and precision, thus contributing to our understanding of the biological fate of macromolecular drugs and biomarkers. A further advantage of LC–MS/MS is that it does not necessarily require immunochemical reagents, which may vary in specificity and quality, and may thus be the cause of a poor comparability of results between and sometimes even within LBA platforms [1]. A limitation, however, is that LC–MS/MS is not easily applicable to intact analytes with molecular masses above 5–10 kDa, mainly because of limited sensitivity [2–4]. Therefore, most quantitative LC–MS/MS assays for larger proteins are based on the enzymatic digestion of the target protein to a series of proteolytic peptides. One or more of these, usually referred to as signature peptides, are subsequently quantified as a surrogate for the intact protein [5–8]. While enzymatic cleavage of a protein analyte typically leads to a higher detection sensitivity because of the better ionization and fragmentation behavior of the resulting peptides, an inherent consequence is that quantitative information is only obtained about a small part of the original protein structure. It is, therefore, important that a signature peptide should be selected that is sufficiently representative for the protein. While most quantitative bioanalytical LC–MS/MS methods for proteins use a single signature peptide, the multiplexing possibilities of the technique allow the straightforward monitoring of multiple peptides from different parts of the protein, and some reports indeed describe the quantification of more than one signature peptide per protein, for various reasons. The aim of this review is to provide an overview of published LC–MS/MS methods for bioanalytical protein quantification, which use two or more signature peptides. Special attention is paid to the purpose of using multiple peptides, such as confirmation of the concentration, monitoring different structural parts or isoforms of proteins or following their *in vivo* biotransformation. It is also summarized

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and discussed whether or not the obtained protein concentrations via the different peptides agree and what the significance is of a possible discrepancy.

Digested protein analysis

For the digestion of proteins, different enzymes can be used. Trypsin is by far most widely used to cleave protein analytes into peptides, because it is widely available, reasonably priced and cleaves the protein chain after the basic amino acids arginine and lysine (except when there is a C-terminal proline), and thus produces positively charged peptides that often have a good mass spectrometric response. Optimal digestion by trypsin occurs at a pH of around 8 and at a temperature of 37°C, whereby a trypsin to protein ratio of 1:20 is commonly used [9]. In case no suitable tryptic signature peptides can be generated, other enzymes such as Lys-C or Glu-C can also be used [10]. Samples can be directly digested in solution [11,12] or the protein fraction, including the target protein, can be isolated by precipitation and centrifugation, followed by digestion of the formed protein pellet [13,14]. If better sensitivity and selectivity are required, digestion can be done after enrichment of the protein analyte from the sample using antibodies [5] or antibody-free sample preparation techniques [15,16]. Where needed, methods include reduction and alkylation steps prior to digestion to break intramolecular disulfide bonds [11,15]. Whatever the approach, all parameters influencing the digestion should be optimized for each of the signature peptides, to ensure a high, reproducible and preferably fast digestion yield, important parameters being trypsin concentration, grade and digestion time [5]. It is very important to use an internal standard during as many sample-preparation steps as possible because, if well chosen, it will correct for variations in each of the steps as well as for fluctuations in the LC–MS/MS analysis. For protein analytes, the ideal internal standard is a stable-isotope labelled (SIL) form of the intact protein, because it is chemically and physically identical to the analyte and, therefore, will also correct for analytical variability when the protein is still intact or being cleaved, during extraction, reduction, alkylation and digestion. Since SIL proteins are often difficult or expensive to obtain, a SIL form of the signature peptide(s) with or without additional amino acids, which are relatively inexpensive and easy to synthesize, are often used as an alternative. If not for all steps of the analysis, a SIL peptide will at least correct for variations during LC–MS analysis [2,17].

Signature peptide selection

Selection of the signature peptide(s) is an essential part of the development of any LC–MS/MS method for a protein analyte. There are a number of analytical and conceptual considerations that play a role for the selection of a proper signature peptide [2,18,19]. First of all, it is crucial that the peptide of choice is unique, that is, that its amino acid sequence does not occur in any non-analyte protein in the matrix of interest, to avoid contributions to the detection response from other, endogenous compounds. Next, to ensure adequate sensitivity and method robustness, signature peptides ideally are of a suitable length (7–20 amino acids) and moderate hydrophobicity, show stable chromatography, and are easily ionized and fragmented. In addition, peptides with potentially unstable amino acids (methionine, asparagine, glutamine) that might lead to degradation during analysis, and amino acid sequences that could lead to missed cleavages, such as multiple successive lysine or arginine moieties and post-transitional modifications, which will reduce proteolytic activity (e.g., acetylation or methylation of lysine, phosphorylation or *O*-glycosylation) [10], are best avoided, or at least require careful experimental optimization [18]. Publicly accessible *in silico* digestion software tools and databases such as mMass [20] and Basic Local Alignment Search Tool (BLAST) [21] can be used to predict the peptides formed after digestion with a particular enzyme as well as their uniqueness in the species of interest. Equally important is considering which part of the protein structure is most relevant for answering a particular research question. For example, when pharmacological activity is related to a specific structural element in a protein, it is important to include that part of the protein in the signature peptide, when a measure for the active concentration is required. When the protein analyte is modified *in vivo*, it may be of interest to specifically include or exclude the modification site in the signature peptide, depending on whether or not the modification impacts drug activity. Altogether, a good understanding of the structure, working mechanism and potential biotransformation of the protein is just as important for signature peptide selection as the analytical properties of the peptide.

Quantification using multiple peptides

A special feature of protein analysis after digestion is that multiple peptides can be selected for quantification and that, therefore, more than one concentration value for the original, intact protein can be obtained. Compared to

Table 1. Overview of the number of peptides used for confirmation.

Analyte	Peptides used (n)	Difference in concentrations found	Reason for the difference found	Ref.
Anterior gradient 2	2	Not discussed	NA	[22]
sRAGE	2	Not discussed	NA	[15,23]
Anti-Factor D	2	Not discussed	NA	[24]
Trastuzumab	2	Not discussed	NA	[25]
Procalcitonin	2	Not discussed	NA	[26]
BMS-986089	2	No	NA	[27]
Ustekinumab	4	Not discussed	NA	[28]
S1PR1	2	Not discussed	NA	[29]
Ricin	2	Not discussed	NA	[30]
Ricin	2	No	NA	[31]
CDH17	3	Not discussed	NA	[32]
Rituximab	2	No	NA	[33]
Human monoclonal antibody drugs	2	No	NA	[34]
mAb-A and mAb-B	2	Not discussed	NA	[35]
Protein 1 (confidential)	2	No	NA	[36]
BMS-A	2	No	NA	[37]
Interleukin 21	2	No	NA	[38]
SPD	2	No	NA	[39]
Protein 1 (confidential)	2	No	NA	[40]
Apolipoproteins	2	Yes	Difference in digestion efficiency	[41]
Alpha-glucosidase	3	No	NA	[8]
Cardiac troponin I	3	No	NA	[42]
SHBG	3	No	NA	[43]
Apolipoprotein A-1 and B	4	No	NA	[44]

NA: Not applicable.

Table 2. Overview of the number of peptides used for discrimination between different protein forms.

Analyte	Peptides used (n)	Difference in concentrations found	Reason for the difference found	Ref.
rhTRAIL	2	Not discussed	NA	[16]
Ranibizumab and bevacizumab	2	Not discussed	NA	[45]
IgG1/IgG2/IgG4	3/3/2	Not discussed	NA	[46]
VDBG	6	Not discussed	NA	[47]
α-lactalbumin, β-lactoglobulin, β-casein, κ-casein, α_{s1}-casein, α_{s2}-casein	2/2/2/2/4/8	Not discussed	NA	[48]
IGF-1	2	Yes	Presence of another IGF-1 isoform	[11]
Human growth hormone (GH) 22 kDa	2	Yes	Presence of another GH isoforms	[5]
Apolipoprotein E (apoE) isoforms	2	Yes	Presence of another apoE isoform	[49]
Amyloid (Aβ)-peptides	2	Yes	Presence of other isoforms	[50]
Progastrin releasing peptide/neuron specific enolase	3/2	Yes	Presence of other isoforms	[51]
Tau protein	2	Yes	Presence of other isoforms	[52]

NA: Not applicable.

small or intact large-molecule analysis, for digested protein quantification there always is the additional question of how many peptides are going to be included in a method, and why. In this section, the different purposes of using multiple peptides are presented and discussed, subdivided into confirmation, discrimination between different protein forms and assessment of biotransformation. Tables 1–3 give an overview of relevant published examples.

Table 3. Overview of the number of peptides used for assessment of biotransformation.

Analyte	Peptides used (n)	Difference in concentrations found	Reason for the difference found	Ref.
Somatropin	3	Yes	*In vivo* effect	[7]
Centyrin	2	No	NA	[53]
Dulaglutide	2	Yes	*In vivo* effect	[54]
Trastuzumab	2	Yes	*In vivo* effect	[6]
Trastuzumab and pertuzumab	2/2	Yes	*In vivo* effect	[13]
Apolipoprotein A-1	9	Yes	*In vivo* effect	[55]

NA: Not applicable.

Confirmation

It is not uncommon that, from the different possible peptides, a primary signature peptide is selected for quantification based on its analytical properties, such as adequate stability and good sensitivity. A second peptide, with somewhat less favorable properties, can then be used for confirmation purposes and to build up knowledge and confidence in the assay. Although many reports include such a confirmatory peptide, it is often not specified how exactly it is used and what actions are taken, if any, in case there are large discrepancies between its concentration and that of the quantifying peptide.

For the quantification of the potential prostate cancer marker AGR2 in human serum and urine, the two best-performing peptides were included in the assay, one of which was used for quantification without mentioning the purpose or the results of the other [22].

Klont *et al.* developed two methods for the quantification of the soluble receptor of advanced glycation end-products (sRAGE) in human serum [15,23]. In both methods, the peptide that performed best in terms of accuracy and precision was selected for quantification and a second peptide was selected to confirm the presence of sRAGE, but no further details were given about how the peptides were compared.

For the quantification of a PEGylated anti-Factor D antigen-binding (Fab) fragment in monkey serum, one peptide from the complementarity-determining region (CDR) of the Fab light chain (LC) was used for quantification and a second peptide from the variable region of the Fab heavy chain (HC) was used for confirmatory and troubleshooting purposes [24]. In a similar way, for the quantification of trastuzumab in human serum [25], a peptide from the variable region of the HC was selected for quantification, based on signal-to-noise and stability considerations, and a monitoring peptide was selected from one of the CDRs of the HC. Although it was stated that the monitoring peptide could be useful for offering insights into digestion efficiency, integrity and biotransformation of the protein, it was not specified how this peptide was used in practice.

An antibody-free method was developed for the quantification of procalcitonin at the pg/ml level in human serum using a quantifying peptide and a peptide for confirmation purposes [26]. The confirmatory peptide was selected because it showed the most sensitivity next to the quantifying peptide. A difference was found between the two peptides in the kinetics of release during digestion, which was attributed to the occurrence of missed cleavages for the confirmatory peptide. This implies that the method was insufficiently optimized for a good comparison of the peptides. Since a peptide-based calibration curve was used, a correction factor was for the quantifying peptide applied to account for the incomplete (38%) protein extraction and digestion. No further details were provided on how the confirmatory peptide was used and how the results compared. It was stated, however, that the quantifying peptide is present in the structure of both intact procalcitonin and an *in vivo* cleavage product, while the confirmatory peptide only occurs in the intact protein, so differences are in fact to be expected.

For the quantification of the fusion protein BMS-986089, two peptides with the highest detection signal were selected, one from the CDR region (the quantification peptide) and the other from the fragment crystallizable (Fc) region (the confirmation peptide) [27]. Both peptides performed well in terms of precision and accuracy, but results were only reported for the primary peptide.

In an immunocapture-LC–MS/MS method for the quantification of ustekinumab and three other monoclonal antibodies (mAbs) in several mouse tissues, multiple peptides with good detection intensity were investigated and four of these were monitored for ustekinumab and two for each of the other mAbs [28]. Apart from mentioning that the use of peptides from multiple regions of the protein allows troubleshooting and evaluating drug stability, no comparison was provided.

For the quantification of S1PR1, a G protein-coupled receptor (GPCR) protein, two peptides from different parts of the protein, were selected [29]. The peptide with the highest sensitivity was selected for quantification of S1PR1 and the second peptide was used for confirmation purposes. Immunocapture with two custom-made antipeptide antibodies was used to extract the two peptides from digested colon biopsy homogenate, but despite this effort to optimize the method for both peptides, no further information about the confirmatory peptide was given.

The toxic protein ricin was quantified in serum by immunocapture and LC–MS/MS, using the two most sensitive peptides out of a total of 19 [30]. Ricin consists of two subunits linked by a disulfide bond and the quantifying peptide was selected from the A-chain, while the confirmatory peptide was from the B-chain, with the quantifying peptide having a tenfold better sensitivity. The presence of both peptides was concluded to indicate that ricin is extracted in its intact form, but it was not specified whether this was actually the case in study samples. In a comparable method for the same protein in beverages such as milk and orange juice [31], the same peptide from the A-chain was chosen for quantification and another confirmatory peptide from the B-chain. The results for both peptides were consistent in spiked samples, with results for bias and precision below 20%, but the method was not applied for the analysis of samples with unknown concentrations.

For the quantification of the protein biomarker CDH17 in tissues, the most sensitive peptide was selected for quantification [32]. Two additional peptides from different regions of the protein were monitored to confirm endogenous levels, but again no further information about comparability of results was provided.

All these examples clearly illustrate that many researchers do not quantitively evaluate the results of confirmatory peptides and there even is not much evidence that they are evaluated at all. If anything, the appearance of a response for such a peptide is seen as a black-or-white confirmation of the presence of the target protein.

Still, there are other reports in which the quantifying and confirmatory peptides are compared in a more quantitative manner. For the quantification of the mAb rituximab in human plasma, two peptides were selected, one from the LC and a second from the HC [33]. In a method validation, CV and bias were below 15% (20% at the lower limit of quantitation) for both peptides. In addition, the difference between the peptide results in individual calibration standards and QC samples was calculated and found to be below 20%. The method was not used to analyze study samples.

Furlong *et al.* [34] reported a generic method for human mAb drugs in serum of animal origin by using two peptides that occur in all human mAbs but that are not present in the endogenous proteins of any of the animal species. One signature peptide was selected from the HC and the other from the LC of the mAbs. The authors suggested that both peptides should be used for quantification and that the percentage difference between the two mAb concentrations thus obtained, relative to the mean, should be less than 20% in at least two thirds of the total number of samples. All results of spiked quality control samples as well as all results from a pharmacokinetic study in monkeys met these criteria, which confirmed the reliability of the data and the *in vivo* structural integrity of the mAb during the 168-h sampling period.

In a combination method for two monoclonal antibodies in human serum by immunocapture and LC–MS/MS [35], next to a quantifying peptide a monitoring peptide was included for each mAb to assess structural integrity and assay robustness. For each sample, a ratio was calculated of the responses obtained for the quantifying and for the monitoring peptide, the response itself being the peak area ratio of the peptide over its internal standard. The CV for this 'ratio of ratios' in the calibrators and quality control samples was well below 30% and it was evaluated for each unknown sample. Significant deviations between values for an unknown sample and the control samples may indicate issues that need further investigation. It was not reported, however, if such issues were indeed encountered.

For the quantification of an unspecified protein with a molecular weight of approximately 150 kDa (protein 1) in monkey plasma, two peptides were selected [36]. The peptide from the active region was used for quantification and a second peptide from the nonactive region was used for confirmatory purposes. The protein 1 concentrations in monkey plasma based on the peptide used for quantification were in good agreement with those obtained with the peptide used for confirmation. The concentration difference between both peptides in 39 out of a total of 41 samples was $<15\%$, with the overall mean difference between both peptides being 0.83% (Figure 1). From the good correspondence between the results of the two peptides it was concluded that protein 1 remained intact *in vivo*, but since the entire plasma protein content was digested we believe this cannot be unequivocally proven: the same consistency would have been obtained if the protein had degraded, as long as both peptide structures remain intact.

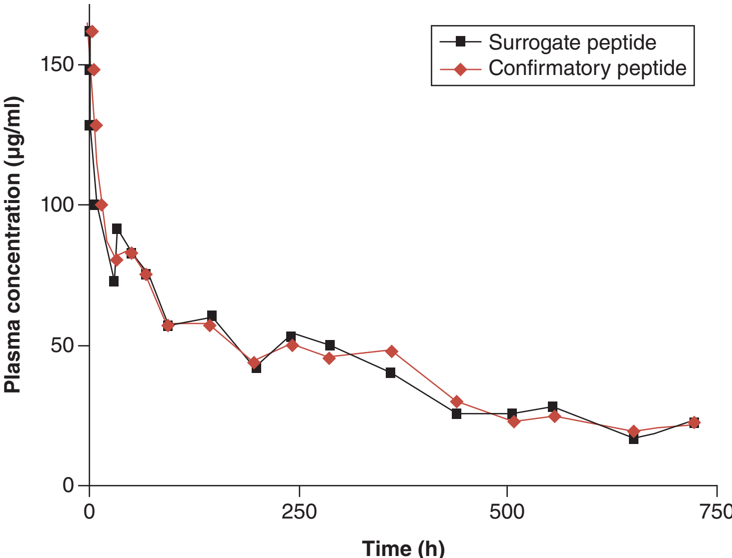

Figure 1. Pharmacokinetic plasma profile in a representative monkey after intravenous administration of protein 1, as derived from two peptides. Reprinted with permission from [36] © Future Science Group (2012).

In a similar approach, two peptides were included for the quantification of protein BMS-A [37]. The validated method showed good accuracy and precision for both peptides and no stability issues were observed. For 90% of all analyzed samples, the difference between the measured concentrations of the two peptides was within 10% of their mean concentration.

Two peptides were used in a method for the quantification of pg/g levels of IL-21 in different human and monkey tissue extracts [38]. One peptide from a section towards the N-terminus was used for quantitation and a second peptide from a section towards the C-terminus was used for confirmatory purposes. A good correlation was observed between the two peptides for all individual human colon and human tonsil IL-21 measurements: the slopes of the correlation curves were within 10% of unity with R^2 values above 0.988.

Surfactant protein D was determined in human serum by immunocapture and LC–MS/MS using two peptides, both located in the C-lectin, ligand-binding domain of the protein [39]. One of the two peptides contains an oxidation-sensitive methionine, which could lead to quantitative differences because of variable degrees of oxidation during analysis. Therefore, an oxidation step was performed prior to digestion to convert all methionines to their oxidized form. Acceptable accuracy and precision were found for both peptides and no stability issues were observed. The method showed excellent interpeptide agreement for 179 clinical samples (Figure 2).

For the quantification of a protein named protein 1 in mouse serum, two signature peptides were selected: peptide-C from the C-domain and peptide-N from the N-domain [40]. The sensitivity of peptide-C was tenfold higher than that of peptide-N owing to better ionization and fragmentation characteristics. For each sample the results were evaluated by assessing the difference between the concentrations of the two signature peptides. Although the lower concentrations could not be quantified by peptide-N, similar data were generally observed for both signature peptides in a 3-h pharmacokinetic profile, with ratios varying between 0.773 and 1.15, indicating that the results from either peptide probably represented intact protein.

For nine different apolipoproteins, the concentrations in simultaneously collected serum and plasma with different anticoagulants were compared [41]. For each protein, a quantifying and a confirmatory peptide were selected. Within each matrix, the two peptides for each of the various apolipoproteins had an average difference of <1.5%, but for two K_2EDTA plasma matrices the individual differences for two of the apolipoproteins were larger, with a negative bias of >14% and a positive bias of >20%, respectively. Investigation of the digestion efficiency versus time profile revealed that this was due to the slower formation of one of the two peptides for these two apolipoproteins in EDTA-plasma.

Occasionally, more than two peptides are included in a comparison. Bronsema *et al.* developed a method for the quantification of the total and antidrug antibody-bound concentrations of the therapeutic protein alpha-glucosidase in human plasma using three different peptides [8]. A peptide from the C-terminal region showed the best analytical performance and was selected for quantification. Two other peptides located near the N-terminus were used for confirmatory purposes. Only the peptide from the C-terminal region was included in the validation

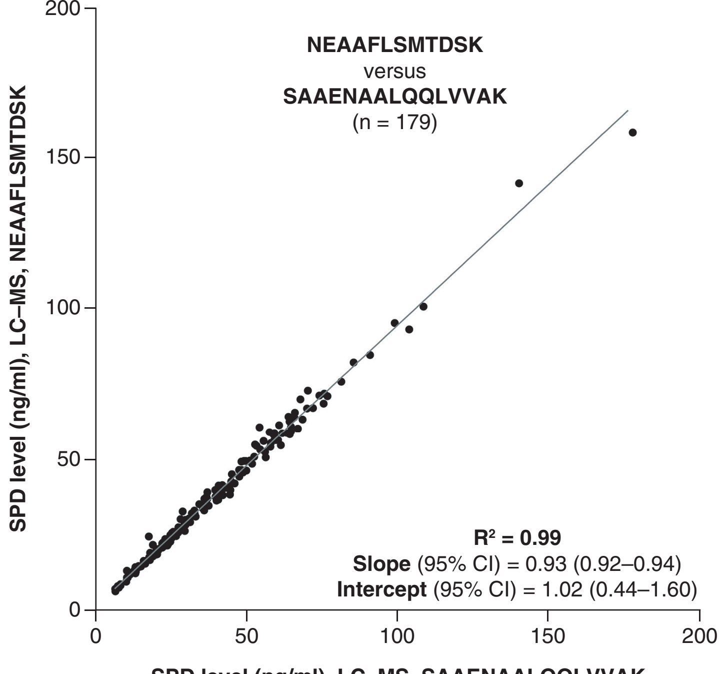

Figure 2. Regression plot of the surfactant protein D concentrations in serum obtained through two tryptic peptides.
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CI: Confidence interval.

of this method, but the other two were also used to determine the protein concentration. Even though the two confirmatory peptides had no internal standards and their concentrations were considered semiquantitative, the average concentration difference between the C-terminal peptide and the two N-terminal peptides was -4.4% and -0.9%, respectively.

The quantification of the biomarker cardiac troponin I human plasma was also performed using three different peptides, although in this case they all had a SIL internal standard [42]. Peptides 1 and 3 were selected because they were located near the stable region of the protein, while peptide 2 lies just outside this stable region. In the five patient samples analyzed, all three peptides correlated well, with coefficients of variation over the found concentrations not exceeding 15%, and peptide 1 and 3 from the stable region showing the best correlation.

Sex hormone-binding globulin was quantified in human serum and plasma using three different signature peptides [43]. These are unique for the protein analyte and also do not occur in the entire rat proteome, which was desirable because of the use of rat serum as a proxy matrix to prepare calibration samples. All three peptides were included in the validation of the method and provided very similar sex hormone-binding globulin concentrations in patient samples, which were between 15.9 and 17.3% lower than the results of a routine immunoassay.

For the quantification of the two apolipoproteins A-1 and B in human serum, five and four different signature peptides were selected, respectively [44]. Although all peptides had favorable analytical properties, their rate of formation and stability during digestion varied considerably, which introduced a source of uncertainty for quantitative use. It was found essential to carefully optimize the digestion time for a good correspondence of the results of the different peptides. Greater variations in interpeptide correlation in the different serum samples were seen after digestion times longer than 4 h.

Altogether, there does not appear to be a lot of consistency within the bioanalytical community about the need for including confirmatory peptides in protein quantification by LC–MS, about how many peptides should be added and about the way the results of the multiple peptides should be evaluated. We believe that monitoring a confirmatory peptide can be helpful in the early part of a method's lifetime, to confirm robustness of the analytical approach but, once more experience has been obtained, quantifying the protein analyte by means of just one signature peptide should be sufficient for reliable results. Including more than one confirmatory peptide may be useful in some cases, such as for large multidomain proteins, but in our opinion would not be necessary as a rule. It is important to realize that it may not always be possible to find a suitable confirmatory peptide or that this peptide may not be able to cover the entire calibration range with sufficient sensitivity. However, if a method includes a second peptide for confirmation, it does make sense to compare and evaluate the results of both peptides. A

comparison such as used for the assessment of incurred sample reproducibility [56], as suggested by [34], would be a logical approach.

Discrimination between different protein forms or species

While similar protein concentrations are typically expected for the different signature peptides in the case of confirmation, there are also situations in which differing concentrations are more likely. Proteins usually are not single, well-defined molecules, but rather families of structurally related protein forms, with differences that can be as small as a single amino acid. Very often, an LBA will not be capable of distinguishing these different forms because of their cross-reactivity in the assay, but the use of LC–MS with multiple peptides that are unique for the different isoforms can typically distinguish these different protein forms within or between species.

For a method for the simultaneous quantification of two forms of recombinant human TNF-related apoptosis-inducing ligand, the wild-type and a mutated form, in human and mouse serum, quantifying peptides were selected with just one amino acid difference [16]. Similarly, confirmatory peptides were included in the assay that differed by one amino acid. The method was used to support a 6-h pharmacokinetic trial in mice, which were simultaneously dosed with both isoforms, and the concentration–time profiles were quite similar.

Bevacizumab, a mAb, and ranibizumab, the corresponding Fab fragment, were quantified together in human plasma using two peptides [45]. One peptide is present in the structures of both bevacizumab and ranibizumab and represents either of the two proteins when dosed alone, or the sum of both, when dosed together. A second peptide was used to distinguish between the treatments with bevacizumab and ranibizumab, because it is only released from ranibizumab after digestion and is therefore absent when patients receive only bevacizumab. It was not used quantitatively because of its lower intensity and less favorable stability, but only for qualitative confirmatory purposes. No further discussion was provided regarding the presence of the second peptide in patient samples.

For the quantification of the IgG1, -2 and -4 versions of an investigational mAb (with 94% sequence homology) in rat serum, each IgG form was represented by multiple unique signature peptides (three, two and two, respectively) [46]. The method was successfully validated and was able to simultaneously quantify IgG1, -2 and -4 using the specific peptides, which was not possible with the ELISA. The method was, however, not used for analysis of samples containing multiple IgG forms, but only for serum obtained after dosing of the IgG4 variant to rats.

For the quantification of the three most abundant isoforms of the vitamin D-binding globulin (VDBG) in human serum, multiple peptides were selected [47]. The measured concentrations of two signature peptides, occurring in each isoform, were averaged to determine the final total concentration of the protein and a third peptide was selected for quality-control purposes. Furthermore, three isoform-specific signature peptides were used to qualitatively distinguish between the VDBG isoforms. A method comparison revealed that the LC–MS/MS method gave significantly higher VDBG concentrations than a routine immunoassay, and that the differences were more pronounced for certain isoforms. This suggests that the immunoassay shows different binding characteristics towards the different isoforms of VDBG.

Twenty different signature peptides from four caseins and two major whey proteins were used for differentiating goat or sheep from cow whey and whole-milk powder in infant formulas [48]. The LC–MS/MS method was successfully applied to samples with different compositions and showed high specificity and accuracy in detection of the species involved.

In some cases, a relatively minor difference in concentration was observed between the selected peptides. In a method for the quantification of the biomarker IGF-1 in human plasma [11], two signature peptides were selected. One of these represents the total IGF-1 concentration, because it occurs in all different isoforms of this protein, while the second peptide includes the N-terminus and is thus present only in the isoforms with an unmodified N-terminal sequence. This will allow discrimination between full-length IGF-1 and a minor variant that has been described, lacking the first three N-terminal amino acids. Accuracy and precision were acceptable for both peptides in samples spiked with full-length IGF-1, but the N-terminal peptide showed on average 8% lower IGF-1 concentrations than the other mid-molecule peptide in 40 clinical samples. This suggests that a small part of plasma IGF-1 has an N-terminal modification, which may be due to the occurrence of the shorter variant.

In a similar way, two signature peptides were selected for the selective quantification of the 22-kDa isoform of the growth hormone GH1 (22 kDa) in serum and plasma [5]. One of the two peptides is unique for GH1 (22 kDa) and thus only represents this specific isoform, while the other occurs in all growth hormone isoforms and provides a total GH concentration. In 44 clinical samples, the total GH concentration was on average 13% higher than the

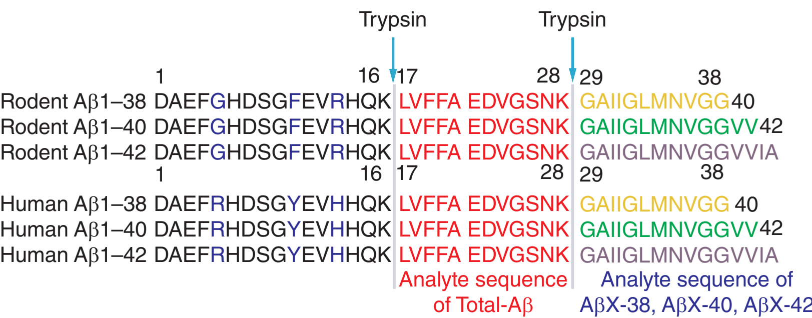

Figure 3. Signature peptides for total-Aβ, Aβx-38, Aβx-40 and Aβx-42, allowing analysis in both humans and rodents.
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isoform-specific concentration, indicating the majority of circulating growth hormone is indeed the GH1 22-kDa variant and that a minor part consists of other isoforms.

There are also reports available where the concentration differences between the selected peptides were more significant. For the quantification of apolipoprotein E (apoE) isoforms in samples from postmortem brain, two peptides were included [49]. One peptide was used for the quantification of total apoE and a second peptide was selected for the specific quantification of the apoE4 isoform, which is speculated to be a risk factor for the development of Alzheimer's disease. The apoE4 isoform was absent in most healthy control samples and detected in the majority of Alzheimer's disease patient samples. There was a good correlation of the apoE4 levels with the available genetic information of the donors: apoE4 was found in all samples corresponding to the ε4 allele and statistically equal to total apoE for the ε4/ε4 genotype, while no apoE4 was detected in all samples not having the ε4 allele.

Three amyloid (Aβ) peptides, which differ in length, were quantified in rat brain tissue [50]. For total-Aβ, a signature peptide was selected that occurs in each form and to distinguish the different isoforms Aβx-38, Aβx-40 and Aβx-42, specific peptides were used (Figure 3), each against their own ^{15}N-labeled internal standard. It remains unclear why a digestion-based approach was selected, since the relatively small size of the peptides would also allow their intact measurement. The method showed acceptable accuracy and precision for total-Aβ, Aβx-38, Aβx-40 and Aβx-42, and for each amyloid (Aβ)-peptide, the concentrations were determined in six rat brain samples. The summated concentrations of the three isoforms were significantly lower than the total-Aβ concentration, which might indicate the presence of yet other isoforms.

A multiplex method for the determination of the small-cell lung cancer markers progastrin-releasing peptide (ProGRP) and neuron-specific enolase (NSE) was developed by Torsetnes *et al.* [51], based on immunocapture with two mAbs and LC–MS/MS. Quantification was performed of two ProGRP isoforms and of total ProGRP, using three different peptides: one peptide represents the total concentration, because it appears in all ProGRP forms, and the other two peptides are unique for each isoform. For NSE two peptides were used, representing the α- and γ-subunits of the γ-, γγ- and αγ-forms of this enzyme. The applicability of the method was shown by the analysis of six patient samples, in which different concentrations were found for total ProGRP and both isoforms, as well as for the α- and γ-subunits of NSE.

To quantify different isoforms of tau protein in human CSF, a quantitative LC–MS/MS method was developed using a signature peptide containing a serine (S396) that is either phosphorylated or not [52]. This peptide is quantified after two different sample treatment workflows, including dephosphorylation and digestion steps. In the first workflow, the form of tau protein that was not originally phosphorylated at S396 is quantified, while the second workflow measures total tau protein, that is, both with and without phosphorylation. The difference in the concentration results between the workflows therefore equals the amount of tau protein originally phosphorylated at S396. The method was successfully used for the analysis of CSF from healthy volunteers and patients, and the percentage phosphorylation on S396 was about 30%.

Biotransformation

LC–MS/MS with multiple peptides can also be used to monitor two or more parts of the same protein simultaneously and, more specifically, to assess whether the concentrations of these different protein parts are diverging as

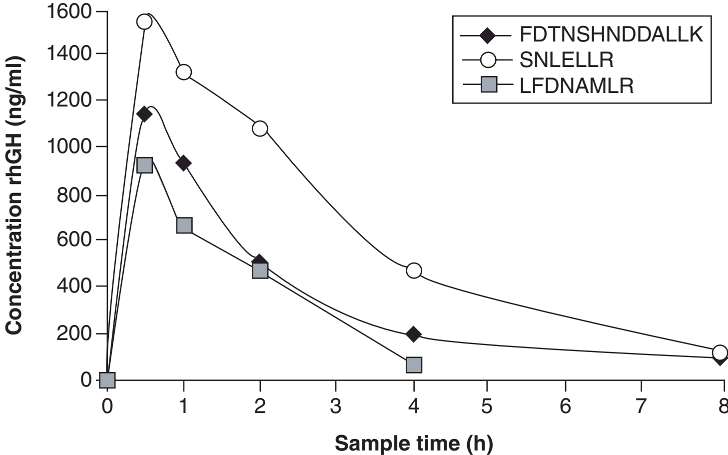

Figure 4. Pharmacokinetic plasma profile in a representative rat after intravenous administration of somatropin, as derived from three peptides.
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a function of sampling time, which could indicate that one of them is being modified *in vivo* by a process usually referred to as biotransformation. Well-known examples are deamidation of asparagine and glutamine, oxidation of methionine and truncation of peptide chains, and these can be followed by selecting signature peptides that include the specific structural element that is subject to biotransformation. Here, it should be noted that the absolute quantification of proteins with a modified structure due to biotransformation is not always straightforward, because pure reference standards of the protein including this structural modification are often not available. As an alternative, quantification could be performed on the peptide level using peptide standards with the specific modified structure.

The number of published applications in this field is relatively limited. Somatropin, the recombinant form of human growth hormone, was quantified in rat plasma using three peptides [7]. The results for accuracy and precision across the relevant concentration range were within regulatory method-validation criteria for all three peptides. Interestingly, in plasma samples of dosed rats, the three signature peptides showed consistently dissimilar concentration results (Figure 4), whereas no differences were observed in spiked samples. The peptide located in the middle of the protein provided higher somatropin concentration results than the other two peptides, which are located closer to the N- and C-terminus of the protein. This suggests an *in vivo* effect, in which the N- and C-terminus of the protein are more susceptible to enzymatic and/or chemical modifications than the mid-part of the protein, but this was not further discussed.

For the quantification of total and conjugated centyrin, a protein–drug conjugate, in plasma and tissue samples two different peptides were selected [53]. One peptide represents conjugated centyrin because it contains the payload and linker, and the second peptide for the quantification of total centyrin was from the unconjugated part of the protein. To evaluate *in vivo* linker stability and tissue distribution, mice were dosed with the protein–drug conjugate. The results for both peptides showed that the levels of total and conjugated centyrin were similar throughout the sampling period, indicating that the linker was stable in circulation.

To monitor the biotransformation of dulaglutide, a glucagon-like peptide 1 (GLP1)-Fc fusion protein, an LC–MS/MS method for its quantification in mouse plasma was developed [54]. Two tryptic peptides were used: an N-terminal peptide from the GLP1 part of the protein containing two possible *in vivo* proteolytic sites and representing unmodified GLP1, and a second peptide located on the Fc portion of the molecule representing the total Fc concentration. After dosing to mice, the total Fc levels measured essentially remained the same after reaching C_{max}, but the levels of the peptide containing the two possible proteolytic sites decreased over time. This indicates that biotransformation and probably proteolytic cleavage is happening *in vivo* to the molecule dulaglutide, particularly between the N-terminus of the GLP1 peptide and the Fc region.

Bults *et al.* described a method for quantitatively monitoring the *in vivo* deamidation of the biopharmaceutical mAb trastuzumab in human plasma using two unmodified signature peptides and three deamidated product peptides [6]. One stable signature peptide represents the total amount of trastuzumab and the second signature peptide, located in the CDR of the HC, contains an asparagine, which is prone to deamidation and therefore represents the (remaining) amount of trastuzumab that has not been deaminated at this particular position. By measuring peptides containing a deamidated succinimide, aspartate or isoaspartate instead of asparagine, it is

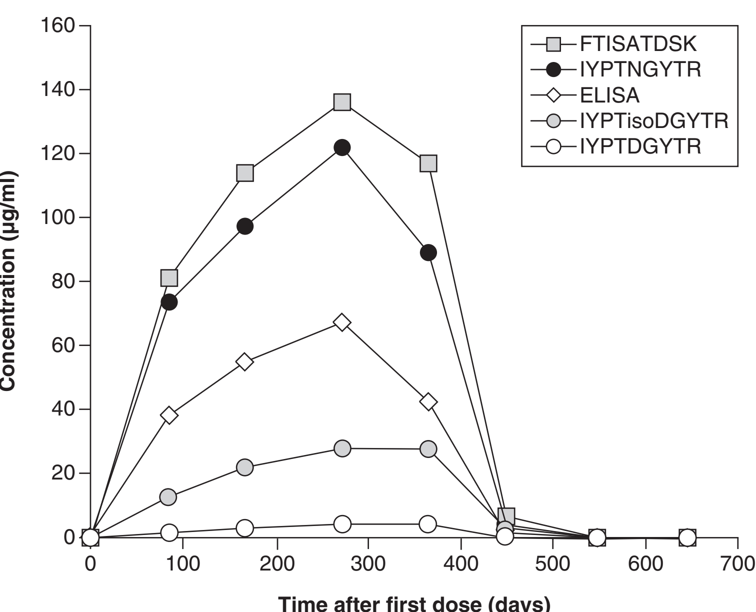

Figure 5. Concentration results for trastuzumab in plasma. Collected from a breast cancer patient on long-term treatment with the drug, obtained by LC–MS/MS for peptides FTISADTSK (total concentration), IYPTNGYTR (concentration with unmodified asparagine), IYPTDGYTR (concentration with asparagine converted to aspartate) and IYPTisoDGYTR (concentration with asparagine converted to isoaspartate).
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possible to assess to which products trastuzumab is converted. Differences between peptide concentrations were observed both in an *in vitro* stress test with trastuzumab spiked into plasma and in patient samples (Figure 5). The deamidation-sensitive peptide showed increasingly lower concentrations than the peptide representing total trastuzumab, and this is due to the conversion of asparagine to isoaspartate and aspartate (but not succinimide), as was confirmed by the appearance of peptides containing these amino acids.

In a similar way, for the quantitative determination of the *in vivo* deamidation of trastuzumab and pertuzumab, two signature peptides of each antibody were selected [13]: one representing the total concentration from a metabolically stable part of the molecule and the other representing the nondeamidated concentration via the peptide containing the deamidation-sensitive asparagine in the heavy chain. A significant difference between the total and nondeamidated concentrations and thus a considerable degree of *in vivo* deamidation was observed in plasma from patients who had been simultaneously dosed with trastuzumab and pertuzumab.

For the quantification of apolipoprotein A-1 in human serum samples from nonsmokers and smokers, nine signature peptides were selected [55]. Excellent correlations of the apolipoprotein A-1 concentrations were found for all nine signature peptides in the samples from 50 nonsmokers and for six of the nine signature peptides in the samples from 50 smokers. For the three other peptides, the correlation was poorer in smokers, which could be due to modifications on these three peptides, such as a chlorination or nitration of a tyrosine, which is supposed to be induced by smoking.

Future perspective

With the increasing importance of biopharmaceutical drugs and protein biomarker research, we believe that LC–MS/MS for protein quantification is here to stay. Although intact protein analysis by LC–MS, typically with high-resolution mass spectrometry, is becoming more popular, digestion-based approaches and quantification of signature peptides will probably remain most widely used, mainly because of its sensitivity advantages. For simplicity reasons, it is understandable that the quantification of just one signature peptide as surrogate for the original protein is an attractive approach. However, obtaining more detailed information by including more peptides in an LC–MS/MS method is relatively easy and we expect that this will be done more often in future, as the experience with proteins and their quantification, as well as the desire for more detailed knowledge about this complicated class of compounds grow. First of all, as a simple means of confirming and increasing the robustness of an LC–MS/MS method and the reliability of the protein concentration results, but in particular also for gaining a better understanding of their structure, possible isoforms and *in vivo* fate. Because of its ability to provide structural information, LC–MS/MS may very well be essential to achieve this goal.

Executive summary

- LC–MS/MS combined with enzymatic digestion has become a mature technique for the quantification of proteins in biological samples as an alternative for or in addition to ligand-binding assays.
- Quantification can be done using multiple peptides.
- In principle, the use of a single signature peptide is sufficient for quantitative purposes, but including multiple peptides adds value for confirmation, to discriminate between different protein forms and to investigate *in vivo* biotransformation.
- The majority of published reports with multiple peptides use one (or occasionally more) additional peptides for confirmation, although it is often not specified how exactly it is used and what conclusions are drawn in case results for the peptides differ.
- Detailed quantitative knowledge about the occurrence of closely related protein forms and about their possible modification *in vivo* can be obtained relatively easily with LC–MS/MS by monitoring multiple peptides, which is a clear advantage over ligand-binding assays.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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